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**TNO report**

**TNO-DV 2006 A268**

**Sleep and Alertness management I:  
Pharmacokinetics of hypnotics and alertness  
enhancers in Marmoset monkeys**

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# Slaap- en alertheidsmanagement I: Farmacokinetiek van slaap- en alertheidsverhogendemiddelen in Marmosetapen



## Probleemstelling

In opdracht van het Ministerie van Defensie wordt door TNO Defensie en Veiligheid onderzoek gedaan naar praktische richtlijnen om tijdens militaire missies ernstige vermoeidheid te voorkomen en de prestaties en alertheid te optimaliseren. Ervaringen met militaire missies hebben namelijk geleerd dat de nadelige effecten van slaapttekort een zeer belangrijke rol speelden bij de uitvoering van de missies. Er zijn farmacologische middelen beschikbaar om deze problemen te voorkomen of op te lossen. Alvorens een advies te kunnen geven over de inzet van dergelijke middelen, is het noodzakelijk meer inzicht te krijgen in de werkingsprofielen ervan. Parallel aan studies in mensen, is het in sommige gevallen nodig dierexperimenteel onderzoek te verrichten naar werkzaamheid en werkingssnelheid en -duur (=farmacokinetiek).

## Beschrijving van de werkzaamheden

In een eerder uitgevoerd onderzoek voor het Ministerie van Defensie is een selectie gemaakt van mogelijk geschikte slaap- en alertheidsverhogende middelen (Busker *et al.*, TNO rapport PML 2000-A2). In het hier gerapporteerde onderzoek is van deze geselecteerde kortwerkende slaapmiddelen temazepam, zolpidem en zaleplon en alertheidsverhogende middelen flumazenil, cafeïne en modafinil de farmacokinetiek in bloedplasma van marmosetaapjes onderzocht. Voor deze farmacokinetische metingen zijn gevoelige en selectieve analyse methodes ontwikkeld die gebruik maken van HPLC- en GC-MS-technieken.

## Resultaten en conclusies

Uit literatuuronderzoek blijkt dat van de slaapinducerende middelen, gemeten in mensen, zaleplon de snelste absorptie en de snelste eliminatie vertoont. Voor de alertheidsverhogende middelen blijkt cafeïne de snelste absorptie te hebben vergeleken met modafinil. De resultaten uit de studie in de marmosetaap laten zien dat ook in deze diersoort zaleplon de snelste opname en eliminatie heeft van de slaapmiddelen en dat van de alertheidsverhogende middelen cafeïne verkiesbaar is boven modafinil met betrekking tot de kinetiek.

Op basis van deze (en andere) gegevens mag geconcludeerd worden dat de marmosetaap een geschikt diersmodel is voor het meten van de farmacokinetische effecten van slaapinducerende en alertheidsverhogende middelen. Bovendien laten de resultaten zien dat zowel in de humane situatie als in het diersmodel op basis van de kinetiek zaleplon en cafeïne mogelijk de meest geschikte middelen zijn voor slaap- en alertheidsmanagement. Echter, dit betekent niet dat deze twee middelen ook het meest effectief zijn. In navolgende rapporten zal het effect op slaap en alertheid in functionele testen besproken worden.

## Toepasbaarheid

Informatie over de kinetiek is zinvol voor de optimalisatie van de effectiviteit van de te gebruiken middelen. Dit specifieke onderzoek is bovendien zinvol voor de extrapolatie van de resultaten naar de effectiviteit van de middelen naar de mens.

## Projectafspraken

Dit onderzoek maakt deel uit van een project waarin de bruikbaarheid van farmacologische middelen voor het optimaliseren van slaap en alertheid wordt onderzocht. In de volgende rapporten zal de invloed van de slaapmiddelen op de normale slaaparchitectuur

## Slaap- en alertheidsmanagement I: Farmacokinetiek van slaap- en alertheidsverhogendemiddelen in Marmosetapen

(TNO-DV 2006 A269; TD2006-057), de invloed van slaapmiddelen op het functioneren  
(TNO-DV 2006 A270; TD2006-058) en de invloed van alertheidsverhogende middelen op het functioneren op momenten dat het circadiane ritme slaap dicteert  
(TNO-DV 2006 A271; TD2006-059) onderwerp van aandacht zijn.

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## Samenvatting

In voorafgaande literatuurstudies zijn aanbevelingen aangedragen welke toegepast kunnen worden binnen de 'crew endurance plans' voor een optimale inzetbaarheid van de militair. Een van deze aanbevelingen was het gebruik van slaapmiddelen en alertheidsverhogende middelen tijdens specifieke missies waarbij men moet presteren op momenten waarbij het circadiane ritme slaap dicteert en zou moeten slapen op tijden waarop men normaal actief is. Echter, elke farmacologische inmenging kan leiden tot ongewenste nevenwerkingen. Daarom heeft het de voorkeur om binnen het slaap- en alertheidsmanagement tijdens militaire operaties gebruik te maken van snelle en kortwerkende slaapmiddelen en snelwerkende alertheidsverhogende middelen. De farmaco-kinetiek van reeds geregistreerde middelen zijn bij mensen over het algemeen goed bekend. Maar deze middelen kunnen mogelijk in een diemodel (marmosetaap) een andere farmaco-kinetiek hebben. Om die reden zal de kinetiek in de marmosetaap vergeleken moeten worden met die in de mens.

Daarom is in deze studie de farmaco-kinetiek van geselecteerde kortwerkende slaapmiddelen temazepam, zolpidem en zaleplon en alertheidsverhogende middelen flumazenil, cafeïne en modafinil in de marmosetaap onderzocht. Dit is van belang om te bepalen of deze middelen voldoen aan de eisen van kort en snel werkend, welke gesteld worden in kort durende scenario's binnen militaire missies.

Om dit doel te kunnen bereiken zijn gevoelige en selectieve methodes ontwikkeld voor monstervoorbereiding en analyse van de bloedmonsters (HPLC en GC-MS).

De bloedmonsters zijn afgenomen met behulp van een stress reducerend protocol, waardoor anesthesie niet nodig is. Dit voorkomt bovendien interactie van het narcosemiddel met de te onderzoeken slaap- en alertheidsverhogende middelen.

Voor het overzicht van de werkingssnelheid, zijn de slaap- en alertheidsverhogende middelen in rangorde geplaatst op basis van de absorptiesnelheid van snel tot langzaam.

	Soort	Absorptie: $t_{cmax}$
Slaapmiddelen	Marmosetapen	ZAL < ZPD < TMP
	Mens	ZAL < ZPD = TMP
Alertheidsverhogende middelen	Marmosetapen	CAF < MOD
	Mens	CAF < MOD

TMP: Temazepam, ZPD: zolpidem, ZAL: zaleplon, CAF: cafeïne, MOD: modafinil.

Uit literatuuronderzoek naar de kinetiek van de geselecteerde slaapmiddelen in de mens blijkt de absorptiesnelheid na zaleplon het snelst te zijn. Voor de alertheidsverhogende middelen blijkt de absorptiesnelheid in de mens van cafeïne sneller te zijn dan die van modafinil. De in dit rapport beschreven resultaten laten zien dat ook in de marmosetaap zaleplon de snelste absorptie vertoont en cafeïne ook sneller werkt dan modafinil.

Uit deze studie blijkt dat zaleplon en cafeïne de meest wenselijke kinetiek vertonen om toe te passen binnen het slaap- en alertheidsmanagement. Bovendien kan geconcludeerd worden dat de marmosetaap een goed model is voor het bepalen van de kinetiek van snelwerkende slaapmiddelen en alertheidsverhogendmiddelen.

Echter, er moet wel op gewezen worden dat de meest optimale kinetiek bij deze twee middelen niet automatisch leidt tot de meest efficiënte effecten op slaap en op alertheid. Daarom zal in de volgende gerelateerde rapporten de effecten op slaapkwaliteit en op gedrag beschreven worden.

## Summary

In earlier studies, possible solutions with direct usefulness in crew endurance plans were recommended; among the recommendations was the use of sleep-inducing and wake-promoting drugs. However, any pharmacological intervention may result in unwanted side-effects. Therefore, for sleep and alertness management in a military setting a combination of a short acting hypnotic drug and a fast acting stimulant drug are preferred. Human pharmacokinetics are usually well-known for approved drugs. However, if these drugs are to be used in a novel animal model setting (the marmoset), pharmacokinetics will be different and need to be re-assessed.

Therefore, in this study, the pharmacokinetics of the short acting hypnotic drugs temazepam, zolpidem and zaleplon, and the alertness enhancers flumazenil, caffeine and modafinil were investigated to determine whether they fulfill the prerequisites of short and fast action to allow the use of these drugs in aiding sleep and enhancing alertness during military service.

In order to attain this goal, sensitive and selective methods of sample preparation and analysis (HPLC and GC-MS) were developed by using high quality separation and detection methods. The blood samples were obtained using a low-stress protocol which makes the use of an anesthetic unnecessary. This also prevents possible intervention with the anesthetic drug with the drugs under investigation.

In order to provide a quick overview of the absorption properties, the drugs are placed in rank order according to their  $t_{Cmax}$  values from quick to slow.

	Species	Absorption: $t_{Cmax}$
Hypnotics	Marmoset monkeys	ZAL < ZPD < TMP
	Human	ZAL < ZPD = TMP
Alertness enhancers	Marmoset monkeys	CAF < MOD
	Human	CAF < MOD

TMP: Temazepam, ZPD: zolpidem, ZAL: zaleplon, CAF: caffeine, MOD: modafinil.

In literature described research in humans showed that, among the selected sleep inducing drugs, zaleplon is absorbed the quickest. Among the tested alertness enhancing drugs, caffeine had a quicker absorption compared to modafinil, in humans. The present study with marmoset monkeys also demonstrated that the absorption of zaleplon is faster than zolpidem or temazepam. Also, the rapid absorption of caffeine compared to modafinil was observed in marmoset monkeys.

Therefore, the present study indicates that zaleplon and caffeine might possess the most favourable pharmacokinetics for sleep- and alertness management.

The present study also shows that the marmoset monkey model is a valid model for measuring the pharmacokinetic effects of fast sleep inducing and alertness enhancing drugs.

However, despite possessing the most favourable pharmacokinetics for sleep- and alertness management, it does not necessarily mean that these two drugs are also the most effective drugs. Therefore, in consecutive reports the effects on sleep quality and the behavioral effects of the selected drugs are discussed.

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A Structural information of target compounds and internal standards

B Materials and chemicals

C Some representative chromatograms of the HPLC and GC-MS analysis of the investigated drugs

# 1 Introduction

Regular and good quality sleep is vital for proper performance and a healthy life. Disturbed sleep is hazardous and can have multiple causes. In a military setting situations that induce disturbed sleep frequently occur: round the clock activity requires rapid work shift changes and long night duties, and provokes sleep loss and high stress levels. This may result in excessive sleepiness. These situations have been documented in a separate TNO report [Simons and Valk, 1999]. Recommended possible solutions with direct usefulness in crew endurance plans included strategic napping, chronobiological treatments and the use of sleep-inducing and wake-promoting drugs.

An overview of sleep-inducing and wake-promoting compounds to aid sleep and to enhance alertness during military service has been given in other TNO reports [Simons and Valk, 1999; Busker *et al.*, 2000]. The report of Busker *et al.* [2000] includes a theoretical evaluation of potential candidates, e.g. hypnotics ('downers') for sleep induction and stimulants ('uppers') for wake maintenance.

Any pharmacological intervention may result in unwanted side-effects. Sleep drugs induce sleep, but may also cause undesired carry-over effects, such as excessive sleepiness after sleep when wakefulness is required. For sleep and alertness maintenance this means that a combination of a short acting hypnotic drug and a fast acting stimulant drug may be necessary in some situations.

To investigate effects of drugs, animal studies are in several respects preferable over human studies. Drug research on human volunteers has practical and ethical problems, and more invasive studies are not possible at all in humans. Therefore, the effects of sleep induction and wake maintenance have been tested in an animal model mimicking human sleep patterns as close as possible: the marmoset monkey.

Key research topics on pharmacological interventions are pharmacodynamics (intrinsic activity of drugs) and pharmacokinetics (level of the active compound at the target site in time). Human pharmacodynamics and pharmacokinetics are usually well-known for approved drugs. However, if these drugs are to be used in a novel animal model setting (the marmoset), pharmacokinetics will be different and need to be re-assessed.

In this study, the pharmacokinetics of the short acting hypnotic drugs temazepam, zolpidem and zaleplon, and the alertness enhancers flumazenil, caffeine and modafinil were determined in the marmoset monkey.

## 2 Background

Information on the physiological function and use of temazepam, zolpidem, zaleplon, caffeine and modafinil has been described extensively by Simons and Valk [1999] and Busker *et al.* [2000], and in the experimental protocols describing the sleep-wake maintenance experiments [Philippens, 2000]. In this chapter relevant pharmacokinetic data of the analyzed drugs in humans are briefly outlined. (see Appendix A for structures and some other details of the investigated drugs).

### 2.1 Temazepam

The most frequently prescribed hypnotic drugs by far are the benzodiazepines. Temazepam is a C<sub>3</sub>-hydroxylated derivative of the benzodiazepine diazepam. It is not very well soluble in water (164 mg/L [Fraschini and Stankov, 1993]). Temazepam easily passes through the blood-brain barrier and its free plasma concentration in humans has been found to correlate well with concentrations in the cerebrospinal fluid.

Temazepam is metabolized into conjugates at the –OH group and is N-demethylated; none of the metabolites appears to be pharmacologically active. About 80% of temazepam entering the body is found as metabolites in urine. Only 2% is excreted unchanged. The plasma elimination half-life in humans is 6-8 hours. Peak plasma levels are achieved between 30 and 50 minutes and elimination has been shown to depend on time of day: at night elimination is faster than during daytime.

### 2.2 Zolpidem

Zolpidem is an imidazopyridine which differs in structure from the benzodiazepines and is a rapid and short acting hypnotic. It is rapidly absorbed after oral administration and is approximately 92% bound to plasma proteins. In humans, the major metabolic routes include oxidation and hydroxylation; none of the metabolites appears to be pharmacologically active. After a single 20 mg oral dose, a peak plasma concentration of 192-324 µg/L occurs 0.75 to 2.6 hours post dose, which extrapolates to a terminal elimination half-life time of 1.5 to 3.2 hours. Zolpidem pharmacokinetics are unchanged during multiple-dose treatment but is influenced by age: clearance in children is 3 times higher than in young adults, and is lower in elderly people.

### 2.3 Zaleplon

The current trend of selecting hypnotics is to use short-half-life agents. The relatively new hypnotic zaleplon is a non-benzodiazepine derivative and was developed to be safer than the earlier developed conventional hypnotics, causing fewer side effects.

After oral administration of 10 mg zaleplon in humans, a maximum plasma level was reached after 0.8-1.5 hour with a maximum concentration of 26 µg/l; the elimination half-life time is 1 hour [Beer *et al.*, 1994; Greenblatt *et al.*, 1998]. Benzodiazepine agonist effects of zaleplon depend on dose and concentration. A bioavailability of 30% was found [Rosen *et al.*, 1999].

Zaleplon shows marked species-dependency in its metabolism. In monkeys, zaleplon is transformed into the 5-oxo-zaleplon rather than into the N-desethyl metabolite [Kawashima *et al.*, 1999]. The latter is the major metabolite in rats and also humans.

## 2.4 Flumazenil

Flumazenil is a specific and competitive antagonist at the central benzodiazepine receptor, reversing all effects of benzodiazepine agonists without tranquillising or anticonvulsant actions [Brogden and Goa, 1991]. As flumazenil appears solely to counteract drugs working on benzodiazepine receptors, its application as alertness-enhancer is limited to use in combination with benzodiazepine(-like) hypnotics.

After oral dosing (200 mg) considerable first pass metabolism was observed, resulting in about 16% of unchanged drug reaching the systemic circulation. Nearly 100% of the drug is cleared by metabolism, mainly by N-desmethylation and acid formation. Elimination half-life is about 1 hour.

## 2.5 Caffeine

Caffeine is a well-known ingredient of alertness enhancing beverages such as coffee and tea. It acts as enhancer of the ability to stay awake at times sleepiness would arise. Caffeine is suggested to act as an antagonist of benzodiazepine receptors [Marangos *et al.*, 1979] and of adenosine receptors.

The plasma peak level is reached within 30-120 min after administration. The concentration of caffeine in plasma in subjects given 230 mg caffeine orally showed a caffeine maximum (6 µg/ml plasma) at 2 h after dosing and an elimination half-life time of about 6 hours [Suzuki *et al.*, 1989].

Data on repeated administration and slow release preparations (SRC, slow release caffeine) indicate that a plasma plateau can be maintained for 4-6 h without overshooting the unwanted effect threshold, which is easily the case after single administration [Beaumont *et al.*, 2004]. Therefore, SRC may be preferred over single administration and is useful to maintain activity in a stable manner.

## 2.6 Modafinil

Modafinil is a wakefulness-promoting agent for oral administration. However, the precise mechanism(s) through which modafinil promotes wakefulness is unknown. Absorption of modafinil tablets is rapid, with peak plasma concentrations occurring at 2-4 hours [Wong *et al.*, 1999].

The major route of elimination (~90%) is metabolism, primarily by the liver, with subsequent renal elimination of the metabolites. Metabolism occurs through hydrolytic deamidation, S-oxidation, aromatic ring hydroxylation, and glucuronide conjugation. Less than 10% of an administered dose is excreted as the parent compound. Only two metabolites reach appreciable concentrations in plasma, i.e., modafinil acid and modafinil sulfone. In preclinical models, modafinil acid, modafinil sulfone, 2-[(diphenylmethyl)sulfonyl]acetic acid and 4-hydroxy modafinil, were inactive or did not appear to mediate the arousal effects of modafinil.

## 3 Materials and methods

### 3.1 Animal model

Sleep in other mammals is in many respects similar to that of humans. Rodent systems, e.g. mice and rats, have however some limitations. Their sleep patterns are poly-phasic and they sleep primarily during the day. This deviates considerably from the consolidated mono-phasic night-time sleep in humans. Sleep patterns with the best resemblance to human sleep are found in primates.

In our laboratory, sleep patterns of marmoset monkeys (*Callithrix jacchus*) have been studied. In Figure 1 photos of marmoset monkeys are shown. These are small primates of about 300-400 g. These animals sleep mono-phasically during the night and have very similar sleep electroencephalogram (EEG) variables, including a sleep intensity decline during the night, regular rapid eye movement (REM) sleep episodes with relatively long cycle duration. Furthermore, experience exists with different read-out systems on behavioral performance. Some of these read-out systems can be used to test the sleep-alertness conditions of the animals.

For pharmacokinetics studies, blood samples of 100-200  $\mu$ l can be taken sequentially from the leg vein (see Figure 1), although the amount of samples is limited (4-5/day). The main problem is finding good veins after other samples have been taken. By sampling strategically around the expected peak time and after the peak, timing of maximal concentration ( $t_{max}$ ), maximal concentration level ( $C_{max}$ ) and elimination half-life ( $t_{1/2}$ ) could be estimated. This estimation is used to find the correct dose and to do behavioral studies when the compound is present in the blood.

In-house very sensitive and selective analytical methods were set up for work-up and analysis of the relatively small marmoset monkey blood and plasma samples. The goal was to determine as low as 5% of  $C_{max}$ . HPLC was the method of choice, except for caffeine where a GC-MS was used which was sensitive enough to allow the use of lower sample volumes.



Figure 1 Marmoset monkey (*Callithrix jacchus*).

For this study adult male marmoset monkeys (*Callithrix jacchus*; see Figure 1), aged 2-6 years with initial body weights between 350-500 g were obtained from Harlan, United Kingdom. The monkeys were housed separately in cages (61 x 61 x 41 cm). The ambient temperature in the housing room was regulated at  $25 \pm 2$  °C and the relative humidity was maintained at  $> 60\%$ . In this room a 12-hour day and night cycle was maintained. However, on the nights of sleep deprivation the light was kept on

during the night. Daily they were fed with pellet chow, peanuts, fruit, boiled egg, baby biscuits, sunflower seeds, bread, beans, and fruit syrup after training or testing.

Water was available *ad libitum*.

All aspects of animal care are described in Standard Operating Procedures, which are in agreement with current guidelines of the European Community. The independent TNO committee on Animal Care and Use approved all protocols for the animal experiments.

### 3.2 Administration and sampling

Drugs were dissolved in a mixture of water and syrup and given orally (2 ml/kg).

For caffeine boiling water was used to make a good solution, modafinil was dissolved only in water.

Blood was taken from the leg, heparinized and centrifuged (14000 rpm, 5 minutes).

The plasma was pipetted off, frozen in liquid nitrogen and stored at -70 °C until analysis work-up. For temazepam determination, whole blood was frozen. Because samples of whole blood give problems with coagulation at work-up, for the other compounds plasma was used.

### 3.3 Materials and chemicals

For the determination of temazepam, zolpidem and zaleplon, TNO standard operating procedures were developed and used [Oostdijk, 2001; Kersten and Oostdijk, 2001, 2002]. In the next paragraphs the used methods are described shortly.

Details on Materials and Chemicals are shown in Appendix B.

### 3.4 Sample clean-up and calibration

#### 3.4.1 Temazepam

##### *Blood Sampling:*

At different sampling times, blood samples (100 – 300 µl) were taken from 4 marmosets. For each sampling time, 30-150 µl blood was used for the analysis.

The first 2 marmosets (pilots) were administrated at a lower dose and could not be used for curve fitting.

##### *Sample preparation:*

Separation of temazepam was done with solid phase extraction (SPE). Heparinized blood (e.g. 150 µl) was mixed with 25 µl MeOH, clonazepam (CZP) was added as internal standard (25 µl 20 µg/ml in MeOH). Water was added to a volume of 1 ml and the mixture was frozen with liquid nitrogen and centrifuged at 14000 rpm for 15 min to remove blood cells. The supernatant was eluted over a pre-conditioned Nexus SPE cartridge and flushed with water. After drying the cartridge, the compounds were eluted with 1 ml methanol and filtered (pore size 0.45 µm). The extract was dried to dryness (vacuum, 30 °C) and dissolved in 120 µl eluents. Samples were analyzed with HPLC within 24 h.

The recovery of temazepam and CZP was in both cases 93% (RSD 2-4%) from spiked plasma (100% using an internal standard). Calibration was performed with a 6 point calibration curve from 0-2.2 µg/ml temazepam in eluents and 2 µg/ml CZP, and showed a good linearity (peakheight ratio,  $r > 0.99$ ). No temazepam was found in blank samples. Because of the same recovery for temazepam and CZP, standards were not worked-up as samples: after evaporation to dryness, eluents were added. The procedure was sensitive

and reproducible: using 150 µl blood, and injection of 50 µl extract (total 120 µl), a detection limit of approximately 10 ng temazepam/ml blood was achieved (S/N 3) [Oostdijk, 2001; Jin *et al.*, 1994; Wolff *et al.*, 1997 and Azzam *et al.*, 1998].

#### 3.4.2 Zolpidem

##### *Blood Sampling:*

At different sampling times, blood samples (30 – 200 µl) were taken from 4 marmosets. Heparinized plasma samples were obtained as described in Paragraph 3.2. For each sampling time 15-50 µl plasma was used for the analysis.

The first marmoset (pilot) was given a higher dose and could not be used for curve fitting.

##### *Sample preparation:*

Separation of zolpidem was done with liquid-liquid extraction (LLE). 50 µl heparinized plasma was mixed with 50 µl MeOH, and 50 µl clobazam (CBZ) (10 µg/ml MeOH) was added as internal standard. 0.5 ml 0.1 M NaOH was added and the mixture was vortexed for 5 sec. 4 ml extraction fluid (45% dichloromethane, 55% n-pentane) was added and vortexed for 30 sec. The mixture was centrifuged (10 min at 4000 rpm), and the organic fraction was pipetted off. The fraction was dried with a rotation-vacuum dryer.

The extract was dissolved in 200 µl eluents for further processing in the HPLC. Samples were analyzed with HPLC within 24 h.

For calibration purposes, 50 µl of blank (human) plasma was used. Calibration was performed with a 7 point calibration curve from 0 - 2.5 µg zolpidem /ml plasma). Further treatment is the same as for the samples. The curves showed a good linearity (peak area ratio,  $r > 0.99$ ). No zolpidem was found in blank samples. The procedure was sensitive and reproducible: using 50 µl plasma, and injection of 40 µl extract (total 200 µl), a detection limit of approximately 10 ng CBZ/ml plasma was achieved (S/N 3). Setting the gain of the detector could lower this limit easily with a factor 10 [Kersten and Oostdijk, 2001].

#### 3.4.3 Zaleplon

##### *Blood Sampling:*

At different sampling times, blood samples (50 – 200 µl) were taken from 4 marmosets. Heparinized plasma samples were obtained as described in Paragraph 3.2. For each sampling time 6-100 µl plasma was used for the analysis.

##### *Sample preparation:*

Separation of zaleplon was done with LLE, following the method of Greenblatt *et al.* [1998]. 50 µl of heparinized plasma was mixed with 10 µl MeOH. 5 µl of zolpidem (20 µg/ml in MeOH) was added as internal standard. 100 µl of Acetonitril (2 time sample volume used) was added to denature proteins. The mixture was centrifuged (10 min at 14000 rpm). The Acetonitril extract fraction was pipetted out and dried in a rotation-vacuum drier. The dried extract was dissolved and homogenized in 250 µl eluents, and filtered (pore size 0.45 µm). Samples were analyzed with HPLC within 24 h.

For calibration purposes, 50 µl of blank (human) plasma was used. Calibration was performed with a 7 point calibration curve from 0 - 15 µg zaleplon/ml plasma). Further treatment is the same as for the samples. The curves showed a good linearity

(peak area ratio,  $r > 0.99$ ). No zaleplon was found in blank samples. The procedure was sensitive and reproducible: using 50  $\mu$ l plasma, and injection of 20  $\mu$ l extract (total 250  $\mu$ l), a detection limit of approximately 5 ng zaleplon per ml plasma was achieved (S/N 3). Setting the gain of the detector and/or less eluents addition could lower this limit easily with a factor 10 [Kersten and Oostdijk 2002; Kersten, 2002; Gubbens-Stibbe, 2001].

#### 3.4.4 Flumazenil

##### *Blood Sampling:*

At different sampling times, blood samples (100 – 250  $\mu$ l) were taken from 2 marmosets. Heparinized plasma samples were obtained as described in Paragraph 3.2. For each sampling time 50-115  $\mu$ l plasma was used for the analysis.

##### *Sample preparation:*

Separation of flumazenil was done with LLE. Heparinized plasma (30-60  $\mu$ l, eg 50  $\mu$ l) of was mixed with phosphate buffer (pH 9.0) to a final volume of 0.60 ml. Next, 10  $\mu$ l MeOH and 20  $\mu$ l clonazepam (100  $\mu$ g/ml) was added and the mixture was vortexed for 5 sec. 2 ml extraction fluid (40% dichloromethane, 60% diethyl ether) was added and vortexed for 30 sec. The mixture was centrifuged (10 min at 3000 rpm), and the organic fraction was pipetted off. The fraction was dried under a gentle stream of nitrogen. The extract was dissolved in 150  $\mu$ l eluents for further processing in the HPLC. Samples were analyzed with HPLC within 24 h.

For calibration purposes, 50  $\mu$ l of blank human plasma was used. Calibration was performed with a 6 point calibration curve from 0 - 40  $\mu$ g flumazenil/ml plasma. Further treatment is the same as for the samples. The curves showed a good linearity (peak area ratio,  $r > 0.99$ ). No flumazenil was found in blank samples. The procedure was reproducible but not so sensitive: using 50  $\mu$ l plasma, and injection of 40  $\mu$ l extract (total 150  $\mu$ l), a detection limit of approximately 50 ng flumazenil/ml plasma was achieved (S/N 3). This method was probably not sensitive enough (see results) or wrong sample times were used; maybe usage of a LC-MS system should be considered for further research. The described method was based on the method of Bun *et al.* [1989].

#### 3.4.5 Caffeine

##### *Blood Sampling:*

A first marmoset (pilot) was given a lower dose and wrong sample times were used. As a result the data from this animal could not be used for curve fitting. After this pilot, blood samples (100 – 300  $\mu$ l) were taken from 4 marmosets after a higher dose at different sampling times. Heparinized plasma samples were obtained as described in Paragraph 3.2. For each sampling time 5  $\mu$ l plasma was used for the analysis. Two other marmosets were administered using a repeated dose regimen to obtain a stable caffeine concentration for a longer time period.

##### *Sample preparation:*

Separation of caffeine was done with solid phase extraction (SPE). Heperanized plasma sample (e.g. 5  $\mu$ l) or 5% BSA for a standard was mixed with 1.5 ml acetic acid buffer (0.1 M, pH 5.0) in a 2 ml micro test-tube (Safe-lock Eppendorf).  $^{13}\text{C}_3$ -Caffeine was added as internal standard (5  $\mu$ l 125  $\mu$ g/ml in MeOH), for standards 0-25  $\mu$ l caffeine was added (131  $\mu$ g/ml in MeOH). Next, the mixture was centrifuged (15 min at 14000 rpm) and eluted over a conditioned Nexus SPE cartridge and flushed with 1 ml acetic acid buffer pH 5.0 and 1 ml water. After drying the cartridge (15 min, vacuum),

the compounds were eluted with 1 ml methanol. For extreme low concentrations the extract can be dried down to dryness and dissolved in 50  $\mu$ l MeOH, although this option was not necessary for the measured samples. Samples were analyzed with GC-MS within 24 h.

Recovery for the method was determined, and 90-110% in the range 6-63 ng. Calibration was performed with a 5 point calibration curve from 0.024 - 3  $\mu$ g caffeine, 5-600  $\mu$ g caffeine/ml plasma). Further treatment is the same as for the samples. The curves showed a good linearity (peak height ratio,  $r>0.99$ ). No caffeine was found in blank samples when using enough washing steps and wash injections after each sample. Because caffeine is widely spread, the use of blank material is advisory. The procedure was reproducible and sensitive: using 5  $\mu$ l plasma, and injection of 1  $\mu$ l extract (total 1 ml), a detection limit of approximately 1  $\mu$ g caffeine per ml plasma was achieved (S/N 3). This detection limit can easily be approved by using a concentration step or by using a larger injection volume, but this seems to be unnecessary because of the high plasma levels analyzed ( $\mu$ g/ml range). The described method was based on the method of Holland *et al.* [1998].

#### 3.4.6 Modafinil

##### *Blood Sampling:*

At different sampling times, blood samples (100 – 200  $\mu$ l) were taken from 6 marmosets. Heparinized plasma samples were obtained as described in Paragraph 3.2. For each sampling time 50  $\mu$ l plasma was used for the analysis.

##### *Sample preparation:*

Sample preparation of modafinil was done by SPE (solid-phase extraction). 50  $\mu$ l of plasma were transferred into 1.5 ml of acetate buffer<sup>1</sup>. After adding 20  $\mu$ l of internal standard solution (10.64  $\mu$ g/ml IPA), the mixture was carefully vortexed and transferred on a Nexus SPE cartridge filled with 200 mg. After washing the cartridge with 1 ml of MQ water, the cartridge was eluted with 2 ml of ethylacetate in a glass tube. After freeze drying in a mixture of acetone and dry ice, the dried ethylacetate was transferred in a HPLC sample vial. The ethylacetate was evaporated to dryness at room temperature with a small stream of nitrogen. After resolving the residue in 200  $\mu$ l of HPLC eluent, the samples were analyzed with HPLC.

##### *Calibration:*

In the same way as the samples, different mixtures with known amounts of modafinil and internal standard were treated with SPE. Also these calibration mixtures were analyzed by HPLC. The curve showed a good linearity (peak height ratio,  $r>0.99$ ). Using 50  $\mu$ l plasma, and injection of 20  $\mu$ l extract (total 200  $\mu$ l), a detection limit of approximately 0.15  $\mu$ g modafinil /ml plasma was achieved (S/N 3). The described method was based on the method of Burnat *et al.* [1998].

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<sup>1</sup> Acetate buffer:

735 mg sodium acetate-trihydrate and 5.4 ml acetic acid (glacial) were dissolved in 500 ml water and adjusted to pH 3.5 with sodium hydroxide (10 M).

### 3.5 HPLC Methods

#### 3.5.1 HPLC configuration 1: temazepam, zolpidem, zaleplon and flumazenil

This HPLC configuration was used for the analysis of temazepam, zolpidem, zaleplon and flumazenil in the sample extracts. Reversed phase HPLC is a useful method for the determination of benzodiazepines [Willems *et al.*, 1985].

The system was configured as shown in Figure 2. It consists of a Shimadzu LC-10AD VP HPLC pump, a pulse damper (SSI, Antec Leyden), an autosampler model 460 (Kontron) with cooled sample compartment, a column-oven (Meta Therm), a UV/VIS detector (Bio-Rad model 1790 UV/VIS detector) and a Fluorescence detector (Jasco intelligent Fluorescence detector model FP-920). Data from both signals could be collected by the data-acquisition system Chromeleon (Dionex, Breda). Samples are cooled at 4 °C in the sample tray. The auto sampler had the option of a variable injection volume from 10-80 µl in a sample loop by using a 100 µl syringe loop injection.

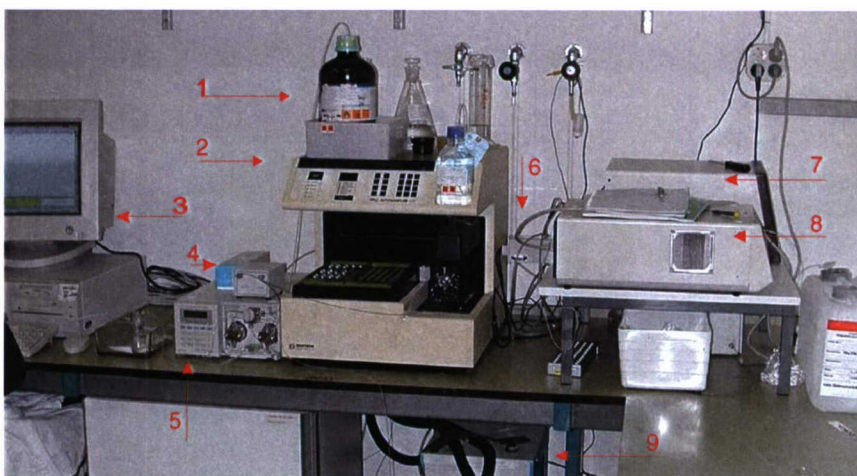


Figure 2 HPLC configuration 1, used for the analysis of temazepam, zolpidem, zaleplon and flumazenil. It consists of: 1) eluent, 2) autosampler 3) PC with data-acquisition software, 4) pulsedamper 5) HPLC pump, 6) column oven, 7) UV/VIS detector, 8) fluorescence detector. Samples in the tray are cooled at 4 °C with a cooler (9).

Conditions from the different HPLC methods are shown in Table 1 and 2.

Table 1 HPLC conditions for configuration 1.

Compound	Column	Eluents	Eluent flow (ml/min)	T (°C)	V <sub>inj</sub> (µL)	Internal standard
TMP	LiChrosfer RP 18	MeOH/ACN/phosphate buffer with 0.5% TEA (pH3,5) (50:10:40) v/v	1.00	35	50	CZP
ZPD	C18 ALLTIMA	phosphate buffer (pH7)/CAN (55:45) v/v	1.00	30	40	CBZ
ZAL	C18 ALLTIMA	phosphate buffer (pH7)/CAN (55:45) v/v	1.00	30	20	ZPD
FMZ	LiChrosfer RP 18	phosphate buffer + TEA (0,5%) v/v (pH7)/CAN (60:40) v/v	1.00	30	40	CZP

TMP: Temazepam; ZPD zolpidem; ZAL zaleplon; FMZ flumazenil; CZP clonazepam; CBZ clobazam  
Columns: guard Inertsil ODS, d<sub>p</sub> 10 µm column, 7.5 mm x 4.6 mm; C18 ALLTIMA column: 150 mm x 4.6 mm, d<sub>p</sub> 5 µm; LiChrospher RP-18, d<sub>p</sub> 3 µm, column: 150 mm x 4.6 mm i.d.; eluents was filtered (0.45 µm) and degassed before use (sparging with helium).

Table 2 Detection parameters of the UV/VIS and fluorescence detector.

Target compound	λ (nm) UV detection	λ (nm) Fluorescence detection	Internal standard	λ (nm) UV detection	λ (nm) Fluorescence detection	Time of wavelength changes (min)
TMP	309	-	CZP	230	-	6.5
ZPD	-	EX: 254 EM: 390	CBZ	230	-	-
ZAL	-	EX: 345 EM: 460	ZPD	-	EX: 254 EM: 390	5
FMZ	245	-	CZP	245	-	-

TMP: Temazepam; ZPD zolpidem; ZAL zaleplon; FMZ flumazenil; CZP clonazepam; CBZ clobazam.

Examples of these chromatograms are shown in Appendix C.

### 3.5.2 HPLC configuration 2: modafinil

In the literature a method is described in detail for the analyses of modafinil by HPLC [Burnat *et al.*, 1998]. The chromatographic conditions were translated to our HPLC configuration 2.

The configuration was used for the analysis of modafinil in the sample extracts.

The system was configured the same as configuration 1. It consists of a Waters model 519 HPLC pump, a Spark autosampler model Basic Marathon, a column-oven model 530 (Alltech), a UV/VIS detector model Spectroflow 757 (Kratos). Data was collected by the data-acquisition system DAX (5.0) and analyzed with version 7.1. A fixed sample loop of 20 µl was installed. The oven was thermostated at 30 °C and UV detection was set at 225 nm. A SunFire C8 5 µm, 4.6 x 150 mm with a SunFire C8 5µm precolumn 4.6 x 20 mm was used for the separation. The buffer consists of Acetonitrile/0.05 M phosphate buffer<sup>2</sup> 28.5/71.5 (v/v) at an eluent flow of 1 ml/min.

An example of a chromatogram, obtained under these conditions is presented in Appendix C.

<sup>2</sup> 0.05 M orthophosphoric acid solution in water was adjusted to pH 2.6 with sodium hydroxide (10 M).

### 3.6 GC-MS method

#### 3.6.1 GC-MS configuration: Caffeine

GC-MS analysis was performed on a HP 5973 mass selective detector which was connected to an Agilent 6890 GC system with a Gerstel MPS-2 auto sampler, using splitless injection (270 °C, 0.25 min splitless time, flow 150 ml/min) on a S/SL injector with a 2 mm tapered Restek liner. The system was operated in the EI mode with a transfer temperature of 220 °C, a source temperature of 230 °C, a quad temperature of 150 °C and an ionization energy of 70 eV. The column used was a RTX-5 fused-silica capillary column (30-m length, 0.25-mm i.d., 1.0 µm film thickness, Restek) or similar. The column flow was set at 2.0 ml/min (helium) constant flow mode. The oven of the chromatograph was kept at 100 °C for 1 min; the temperature was then programmed to 260 °C at 20 °C/min and subsequently held at this temperature for 10 min. Injection volume was 1 µl.

Selected Ion Monitoring (SIM) chromatograms were recorded (6.5-16 min) after monitoring for  $m/z$  194 (T, Target) and 109 (Q, qualifier) (caffeine) and  $m/z$  197 (T) and 111 (Q) for the internal standard  $^{13}\text{C}_3$ -caffeine (dwell time 15 msec). Because of interfering peaks at  $m/z$  194, calculations were based at peak area (peak height if there was an impurity not completely separated from target compound) ratio of  $m/z$  194 and 197 of the  $\text{C}_{12}/\text{C}_{13}$ -caffeine. The T/Q ratio was used as extra identification, but not used for quantification.

An example of a chromatogram is shown in Appendix C.

## 4 Results

Results are presented as average time and drug concentration in plasma and data are fitted with a two compartment model as a bi-exponential Equation (1) with Table Curve.

$$[C]_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \quad (1)$$

This fitting is only an estimation since the small amount of animals and data points per sample time, do not provide enough information to obtain a precise result.

When possible, weight factors are applied: after calculating the standard error of mean (s.e.m.)<sup>3</sup> (2) the weight factor *f* was calculated (3) or when no standard deviation was available, (1/value) was used. Weight factors were not used when the fitted curve was far from all points, for e.g. when only some points have a very large *f*, the fitting was not correct.

From Equation (1) the  $C_{\max}$ , time of  $C_{\max}$  and  $t_{1/2}$  elimination was calculated or derived (4).

$$s.e.m. = \frac{S}{\sqrt{n}} \quad (2)$$

$$f = \frac{1}{(s.e.m.)^2} \text{ or } f = \frac{1}{value} \quad (3)$$

$$t_{1/2,elimination} = \frac{\ln 2}{\beta} \quad (4)$$

Real sampling times are averaged and mean was used. Other toxicokinetic parameters can be calculated, but this was not relevant for our research goal. Also, due to the limited number of animals used and sample time points, the observed kinetic parameters can be considered to be only an estimation.

### 4.1 Temazepam

The amounts of temazepam (averaged) calculated as µg/ml blood at different sampling times, measured after sample preparation as described in Paragraph 3.4.1, and under the HPLC conditions, as described in Paragraph 3.5.1, are presented in Table 3 and 4. The mean blood concentration-time course of temazepam is shown in Figure 3.

In Table 3 the results of the pilot experiment at a lower dose (5 mg/kg) are presented. From these results (and behavior experiments) new sample times and dose were chosen.

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<sup>3</sup> The SEM is used to calculate confidence intervals for the mean. When the distribution of the observations is Normal, or approximately Normal, and the sample size is large, then there is 95% confidence that the population mean is located in the interval  $\pm 1.96$  SEM. If the sample size is less than 30 then the multiplier is not 1.96, but is taken from the t-distribution with *n*-1 degrees of freedom and a confidence of 95% (see table Values of the t-distribution). The 95% confidence interval is calculated by  $\pm t$  SEM.

The mean concentration-time course shown in Figure 3 was fitted to a bi-exponential equation, containing an adsorption phase and elimination phase. Toxicokinetic parameters derived (estimation) from the bi-exponential fit (15 mg/kg) are presented in Table 5.

Table 3 Concentration ( $\mu\text{g/ml}$ ) of temazepam in blood of marmosets after oral administration of a dose of 5 mg/kg (pilot experiment). Mean values with SEM are also presented.

Time (min)	a: b:	1 359	2 381	Mean	SEM	n
0		b.d.	b.d.	0	0	2
37		0.258	0.323	0.29	0.032	2
91		n.d.	0.340	0.34	-	1
112		0.138	n.d.	0.138	-	1
120		0.155	n.d.	0.155	-	1
244		0.075	0.195	0.135	0.060	2
1434		b.d.	0.025	0.013	0.013	2

*a* = animal number, *b* = weight of the animal (g), *b.d.* = below detection limit (0 was used in average), *n.d.* = not determined.

New experiments were performed with a higher dose and other sample times. These are shown in Table 4.

Table 4 Concentration ( $\mu\text{g/ml}$ ) of temazepam in blood of marmosets after oral administration of a dose of 15 mg/kg. Mean values with SEM are also presented.

Time (min)	a: b:	1 350	2 388	3 412	4 327	Mean	SEM	n
0		b.d.	0.002	b.d.	b.d.	0.00	0	4
29		0.302	0.278	0.162	n.d.	0.247	0.043	3
37		n.d.	n.d.	n.d.	0.528	0.528	-	1
60		0.403	0.308	0.365	0.601	0.419	0.064	4
94		0.508	0.368	0.345	0.463	0.421	0.039	4
122		0.538	0.432	0.247	n.d.	0.405	0.085	3
234		0.191	0.121	0.090	0.070	0.118	0.027	4

*a* = animal number, *b* = weight of the animal (g), *b.d.* = below detection limit (0 was used in average), *n.d.* = not determined.

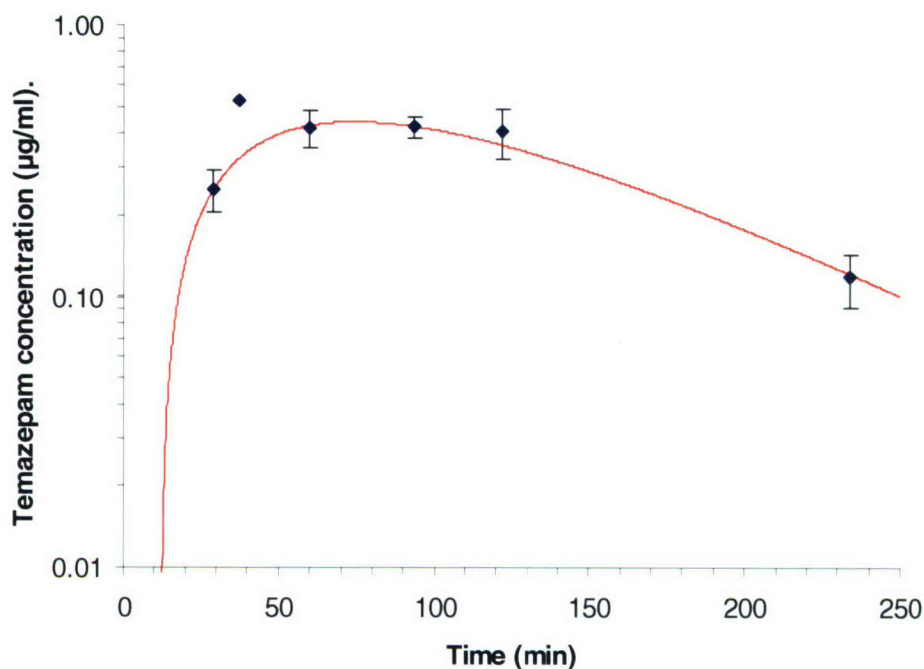


Figure 3 Mean concentration-time course of temazepam (µg/ml, ± SEM, for values of n see Table 4) in blood of marmosets after oral administration of a dose of 15 mg/kg.

Table 5 Toxicokinetic parameters<sup>a, b, c</sup> of temazepam in marmosets after oral administration of a dose of 15 mg/kg.

Parameter	Dimension	Value
Dose	µg.kg <sup>-1</sup>	15,000
A	µg/ml	-11.6
B	µg/ml	11.3
α	min <sup>-1</sup>	0.0171
β	min <sup>-1</sup>	0.0151
t <sub>1/2, β</sub>	min	46
C <sub>max</sub>	µg/ml (blood)	0.44 (≈0.22 in plasma)
t <sub>Cmax</sub>	min	74

a The concentration of temazepam at time t is  $[C]_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$ .

b t<sub>1/2, β</sub>: half-life time of the second toxicokinetic phase (elimination).

c r<sup>2</sup> of Curve fit was 0.99.

## 4.2 Zolpidem

The amounts of zolpidem calculated as µg/ml plasma at different sampling times (averaged), measured after sample preparation as described in Paragraph 3.4.2, and under the HPLC conditions, as described in Paragraph 3.5.1, are presented in Table 6 and 7. The mean plasma concentration-time course of zolpidem is shown in Figure 4.

In Table 6 the results of the pilot experiment at a higher dose (approximately 5 mg/kg) are presented. From these results (and observations) new sample times and dose were chosen.

The mean concentration-time course shown in Figure 4 was fitted to a bi-exponential equation, containing an adsorption phase and elimination phase. Toxicokinetic parameters derived (estimation) from the bi-exponential fit (3 mg/kg) are presented in Table 8.

Table 6 Concentration ( $\mu\text{g/ml}$ ) of zolpidem in plasma of a marmoset after oral administration of a dose of 5 mg/kg (pilot experiment).

Time (min)	a: b:	1 477
0		b.d.
36		1.16
65		0.499
129		0.496
250		0.250

$a$  = animal number,  $b$  = weight of the animal (g),  $b.d.$  = below detection limit.

New experiments were performed with a higher dose and other sample times. These are shown in Table 7.

Table 7 Concentration ( $\mu\text{g/ml}$ ) of zolpidem in plasma of marmosets after oral administration of a dose of 3 mg/kg. Mean values with SEM are also presented.

Time (min)	a: b:	1 428	2 492	3 436	4* 411	5 460	Mean	SEM	N
0		b.d.	b.d.	b.d.	b.d.	b.d.	0	0	4
6		n.d.	0.015	n.d.	n.d.	n.d.	0.015	-	1
12		0.070	n.d.	n.d.	n.d.	n.d.	0.070	-	1
20		0.261	0.065	0.116	0.032	n.d.	0.147	0.059	3
41		n.d.	0.478	0.197	0.036	0.448	0.374	0.089	3
62		0.391		0.550	0.050	0.293	0.411	0.075	3
102		n.d.	0.187	0.368	n.d.	0.255	0.270	0.053	3
120		n.d.		0.253	0.099	n.d.	0.253	-	1
246		0.066		0.235	0.042	0.144	0.148	0.049	3

$a$  = animal number,  $b$  = weight of the animal (g),  $b.d.$  = below detection limit (0 was used in average),  $n.d.$  = not determined, \* animal excluded because of vomiting after oral zolpidem administration.

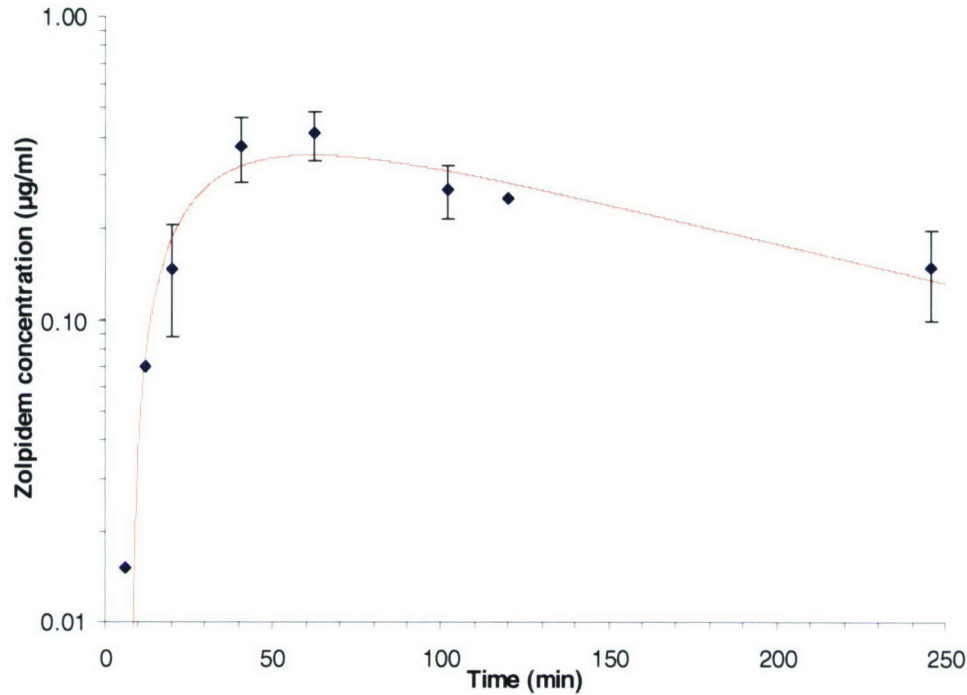


Figure 4 Mean concentration-time course of zolpidem (µg/ml, ± SEM, for values of n see Table 7) in plasma of marmosets after oral administration of a dose of 3 mg/kg.

Table 8 Toxicokinetic parameters<sup>a, b, c</sup> of zolpidem in marmosets after oral administration of a dose of 3 mg/kg.

Parameter	Dimension	Value
Dose	µg.kg <sup>-1</sup>	3,000
A	µg/ml	-0.79
B	µg/ml	0.59
α	min <sup>-1</sup>	0.0427
β	min <sup>-1</sup>	0.00594
t <sub>1/2, β</sub>	min	117
C <sub>max</sub>	µg/ml (plasma)	0.35
t <sub>Cmax</sub>	min	62

a The concentration of zolpidem at time t is  $[C]_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$ .

b t<sub>1/2, β</sub> : half-life time of the second toxicokinetic phase (elimination).

c r<sup>2</sup> of Curve fit was 0.87.

### 4.3 Zaleplon

The amounts of zaleplon calculated as µg/ml plasma at different sampling times (averaged), measured after sample preparation as described in Paragraph 3.4.3, and under the HPLC conditions, as described in Paragraph 3.5.1, are presented in Table 9. The mean plasma concentration-time course of zaleplon is shown in Figure 5.

The mean concentration-time course shown in Figure 5 was fitted to a bi-exponential equation, containing an adsorption phase and elimination phase.

Toxicokinetic parameters derived (estimation) from the bi-exponential fit are presented in Table 10.

Table 9 Concentration (µg/ml) of zaleplon in plasma of marmosets after oral administration of a dose of 10 mg/kg. Mean values with SEM are also presented.

Time (min)	a: b:	1 427	2 507	3 418	4 376	Mean	SEM	n
0		b.d.	b.d.	b.d.	b.d.	0	0	4
10		n.d.	0.068	n.d.	n.d.	0.068	-	1
19		0.238	n.d.	n.d.	n.d.	0.238	-	1
26		n.d.	0.247	n.d.	n.d.	0.247	-	1
42		0.229	0.303	0.185	0.511	0.307	0.072	4
65		0.252	n.d.	n.d.	n.d.	0.252	-	1
105		n.d.	n.d.	0.172	0.440	0.306	0.134	2
125		0.104	n.d.	n.d.	n.d.	0.104	-	1
183		n.d.	n.d.	0.032	0.039	0.036	0.004	2
249		n.d.	0.025	0.023	0.012	0.020	0.004	3

a = animal number, b = weight of the animal (g), b.d. = below detection limit (0 was used in average), n.d. = not determined.

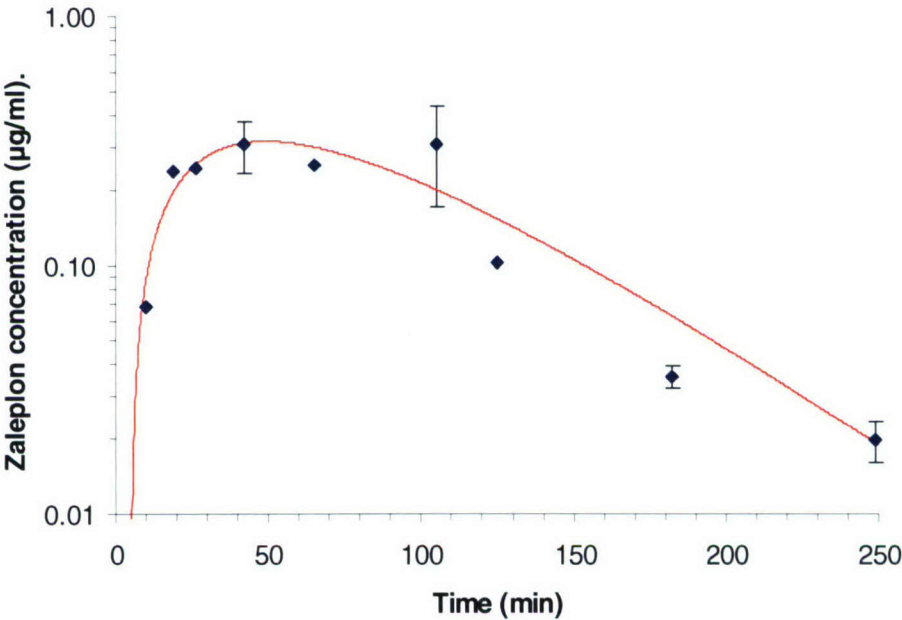


Figure 5 Mean concentration-time course of zaleplon (µg/ml, ± SEM, for values of n see Table 9) in plasma of marmosets after oral administration of a dose of 10 mg/kg.

Table 10 Toxicokinetic parameters<sup>a, b, c</sup> of zaleplon in marmosets after oral administration of a dose of 10 mg/kg.

Parameter	Dimension	Value
Dose	µg.kg <sup>-1</sup>	10,000
A	µg/ml	-6.94
B	µg/ml	6.83
α	min <sup>-1</sup>	0.0242
β	min <sup>-1</sup>	0.0211
t <sub>1/2, β</sub>	min	33
C <sub>max</sub>	µg/ml (plasma)	0.32
t <sub>Cmax</sub>	min	49

<sup>a</sup> The concentration of zaleplon at time *t* is  $[C]_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$ .

<sup>b</sup> t<sub>1/2, β</sub>: half-life time of the second toxicokinetic phase (elimination).

<sup>c</sup> r<sup>2</sup> of Curve fit was 0.83.

#### 4.4 Flumazenil

The amounts of flumazenil calculated as µg/ml plasma at different sampling times (averaged), measured after sample preparation as described in Paragraph 3.4.4, and under the HPLC conditions, as described in Paragraph 3.5.1, are presented in Table 11.

The mean concentration-time course could not be fitted due to a lack of data. Maybe the flumazenil sampling times should be taken later (>100 min) or a more sensitive technique, like LC-MS should be used. A short test to analyze the samples on GC-MS/PCI failed because of the thermal instability of the compound (data not shown).

Peaks in the analyzed samples from the first animal where not optimal (peaksplitting, see Appendix C), maybe due to contamination during the sample-clean up. This occurred in both the samples as well as in the analyzed standard, which allowed for at least some comparison and conclusions.

Table 11 Concentration (µg/ml) of flumazenil in plasma of marmosets after oral administration of a dose of 15 mg/kg. Mean values with SEM are also presented.

Time (min)	a: b:	1 348	2 403	Mean	SEM	n
0		b.d.	b.d.	0	0	2
7		n.d.	b.d.	0	-	1
13		b.d.	n.d.	0	-	1
23		b.d.	b.d.	0	0	2
60		0.73	b.d.	0.37	0.37	2
108		b.d.	n.d.	0	-	1
126		1.23	n.d.	1.23	-	1

*a* = animal number, *b* = weight of the animal (g), *b.d.* = below detection limit (0 was used in average), *n.d.* = not determined.

#### 4.5 Caffeine

The amounts of caffeine calculated as µg/ml plasma at different sampling times (averaged), measured after sample preparation as described in Paragraph 3.4.5, and

under the GC-MS conditions, as described in Paragraph 3.6.1, are presented in Table 12-14.

The mean plasma concentration-time course of caffeine is shown in Figure 6 and 7.

In Table 12 the results of the pilot experiment at a lower dose (15 mg/kg) are presented. From these results (and behavior experiments) new sample times and dose were chosen.

The mean concentration-time course shown in Figure 6 was fitted to a bi-exponential equation, containing an adsorption phase and elimination phase. In Figure 7 the combined data of 30 mg/kg and repeated caffeine dosing was shown. Toxicokinetic parameters derived (estimation) from the bi-exponential fit (30 mg/kg) are presented in Table 15

Table 12 Concentration ( $\mu\text{g/ml}$ ) of caffeine in plasma of a marmoset after oral administration of a dose of approx. 15 mg/kg (pilot experiment, dose uncertain due to vomiting).

Time (min)	a: b:	1 370
0		0.04
6		4.3
11		5.1
19		5.8
30		6.6

$a$  = animal number,  $b$  = weight of the animal (g).

New experiments were performed with a higher dose and other sample times. These are shown in Table 13.

Table 13 Concentration ( $\mu\text{g/ml}$ ) of caffeine in plasma of marmosets after oral administration of a dose of 30 mg/kg. Mean values with SEM are also presented.

Time (min)	a: b:	1 470	2 472	3 388	4 452	Mean	SEM	n
0		b.d.	n.d.	b.d.	b.d.	0	0	3
6		n.d.	n.d.	7.82	n.d.	7.82	-	1
14		14.1	4.5	n.d.	7.90	8.83	2.81	3
28		16.5	4.4	18.3	8.72	12.0	3.27	4
66		16.3	6.0	26.2	10.0	14.6	4.39	4
137		20.6	5.2	27.1	9.55	15.6	5.02	4
285		n.d.	n.d.	21.2	7.57	14.4	6.80	2
1445		n.d.	n.d.	2.99	1.80	2.39	0.60	2

$a$  = animal number,  $b$  = weight of the animal (g),  $b.d.$  = below detection limit (0 was used in average),  $n.d.$  = not determined.

To get a longer and more stable caffeine concentration (and effect), like a slow release preparation of caffeine, a repeated dose experiment was carried out with 2 marmosets. At 0 min, 30 mg/kg as given, next at 4 hours 5 mg/kg and at 8 hours, 10 mg/kg was given orally. Results are presented in Table 14.

Table 14 Concentration ( $\mu\text{g/ml}$ ) of caffeine in plasma of marmosets after a repeated oral administration of a dose of 30 mg/kg, 5 mg/kg (4 h) and 10 mg/kg (8 h). Mean values with SEM are also presented.

Time (min)	a: b:	1 *	2 *	Mean	SEM	n
0		b.d.	b.d.	0	0	2
234		7.01	3.99	5.50	2.14	2
306		10.11	5.34	7.73	3.37	2
474		7.24	4.01	5.63	2.28	2
540		10.52	8.91	9.72	1.14	2
722		8.69	6.46	7.58	1.58	2
1098		2.86	2.52	2.69	0.24	2
1439		2.10	2.00	2.05	0.071	2

*a* = animal number, *b* = weight of the animal (was not documented \*) (g), *b.d.* = below detection limit (0 was used in average), *n.d.* = not determined.

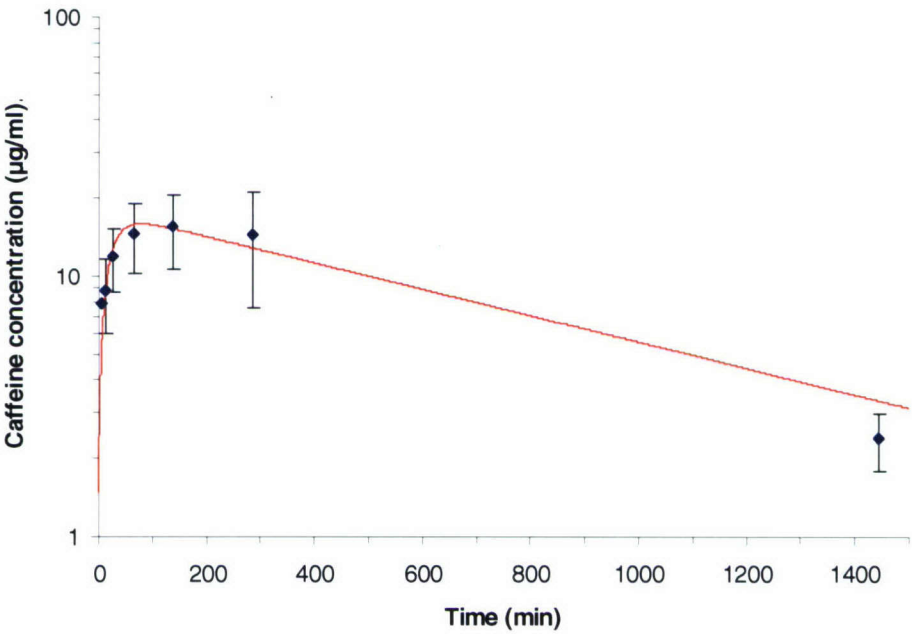


Figure 6 Mean concentration-time course of caffeine ( $\mu\text{g/ml}$ ,  $\pm$  SEM, for values of *n* see Table 13) in plasma of marmosets after oral administration of a dose of 30 mg/kg.

Table 15 Toxicokinetic parameters<sup>a, b, c</sup> of caffeine in marmosets after oral administration of a dose of 30 mg/kg.

Parameter	Dimension	Value
Dose	$\mu\text{g}\cdot\text{kg}^{-1}$	30,000
A	$\mu\text{g}/\text{ml}$	-16.5
B	$\mu\text{g}/\text{ml}$	18.0
$\alpha$	$\text{min}^{-1}$	0.0466
$\beta$	$\text{min}^{-1}$	0.00116
$t_{1/2, \beta}$	min	595
$C_{\max}$	$\mu\text{g}/\text{ml}$ (plasma)	16
$t_{C_{\max}}$	min	79

a The concentration of caffeine at time  $t$  is  $[C]_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$ .

b  $t_{1/2, \beta}$ : half-life time of the second toxicokinetic phase (elimination).

c  $r^2$  of Curve fit was 0.94

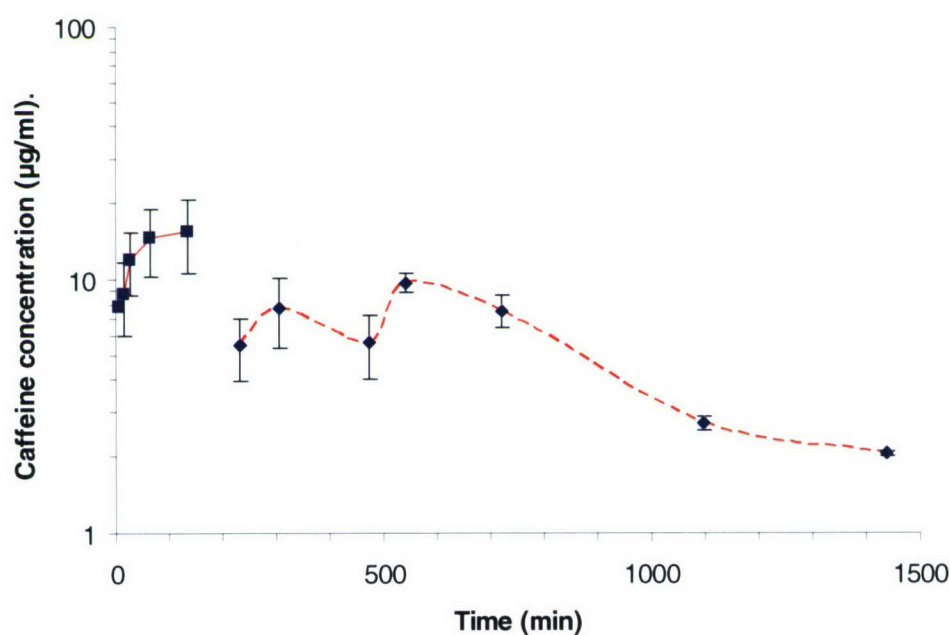


Figure 7 Mean concentration-time course of caffeine ( $\mu\text{g}/\text{ml}$ ,  $\pm$  SEM, see Table 14) in plasma of two marmosets after oral administration of a dose of 30 mg/kg (solid line) and repeated dose of 5 mg/kg at 4 h and 10 mg/kg at 8 h (dashed line).

#### 4.6 Modafinil

The amounts of modafinil calculated as  $\mu\text{g}/\text{ml}$  plasma at different sampling times (averaged), measured after sample preparation as described in Paragraph 3.4.6, and under the HPLC conditions, as described in Paragraph 3.5.2, are presented in Table 16. The mean plasma concentration-time course of modafinil (100 mg/kg) is shown in Figure 8.

The mean concentration-time course shown in Figure 8 was fitted to a bi-exponential equation, containing an adsorption phase and elimination phase. Toxicokinetic parameters derived (estimation) from the bi-exponential fit are presented in Table 17.

There were not enough data points in the last part to use a tri-exponential equation. Therefore the goodness of fit of the curve was not so high. The last part of the curve seems to have another slope as after the top.

Table 16      Concentration (µg/ml) of modafinil in plasma of marmosets after oral administration of a dose of 100 mg/kg. Mean values with SEM are also presented.

Time (min)	a: b:	1 406	2 402	3 408	4 390	5 415	6 390	Mean	SEM	n
0		b.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.00	-	1
9		1.57	n.d.	n.d.	n.d.	n.d.	n.d.	1.57		1
21		1.97	1.07	0.910	0.980	0.390	0.890	1.03	0.21	6
71		9.38	2.04	13.9	12.0	2.22	3.82	7.24	2.13	6
141		23.0	n.d.	n.d.	n.d.	n.d.	n.d.	23.0	-	1
281		28.4	1.03	5.17	10.9	2.79	3.97	8.71	4.17	6
420		285*	0.910	4.57	6.38	1.20	2.75	7.38	4.30	6
776		18.8*	0.380	2.15	2.55	0.150	1.24	4.21	2.94	6
1433		4.64	0.200	1.27	n.d.	b.d.	0.430	1.31	0.86	5

a = animal number, b = weight of the animal (g), b.d. = below detection limit (0 was used in average), n.d. = not determined, \* = value obtained from intrapolation of a curvefit on data of this one animal.

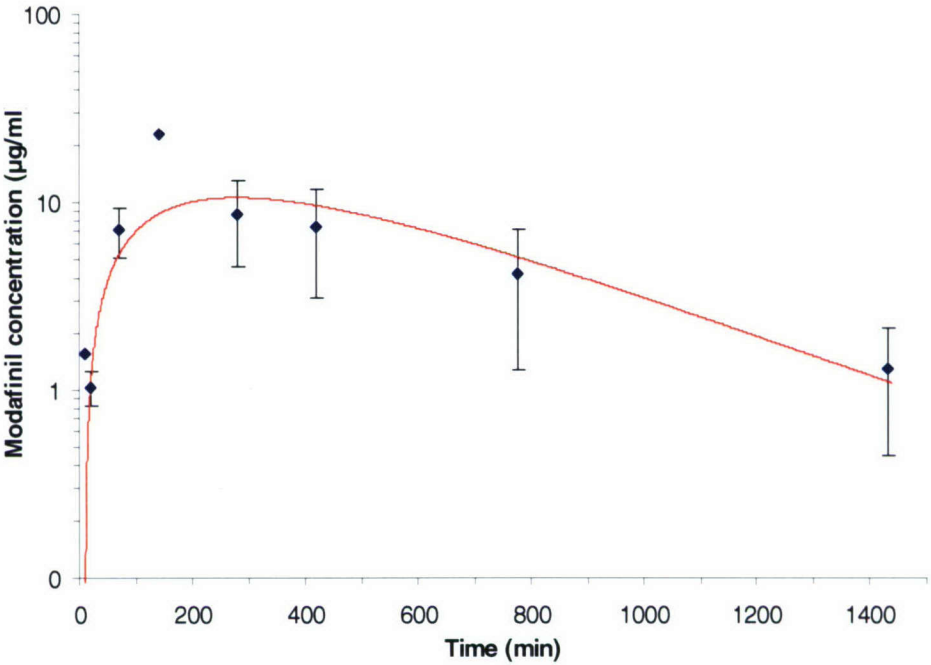


Figure 8      Mean concentration-time course of modafinil (µg/ml, ± SEM, for values of n see Table 16) in plasma of marmosets after oral administration of a dose of 100 mg/kg.

Table 17 Toxicokinetic parameters<sup>a, b, c</sup> of modafinil in marmosets after oral administration of a dose of 100 mg/kg.

Parameter	Dimension	Value
Dose	$\mu\text{g}\cdot\text{kg}^{-1}$	100,000
A	$\mu\text{g}/\text{ml}$	-42.1
B	$\mu\text{g}/\text{ml}$	40.9
$\alpha$	$\text{min}^{-1}$	0.00527
$\beta$	$\text{min}^{-1}$	0.00250
$t_{1/2, \beta}$	min	277
$C_{\text{max}}$	$\mu\text{g}/\text{ml}$ (plasma)	10.7
$t_{\text{Cmax}}$	min	279

<sup>a</sup> The concentration of modafinil at time  $t$  is  $[C]_t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t}$ .

<sup>b</sup>  $t_{1/2, \beta}$ : half-life time of the second toxicokinetic phase (elimination).

<sup>c</sup>  $r^2$  of Curve fit was 0.84.

## 5 Discussion

In earlier studies, possible solutions with direct usefulness in crew endurance plans were recommended; among the recommendations was the use of sleep-inducing and wake-promoting drugs [Busker *et al.*, 2000; Simons and Valk, 1999]. However, any pharmacological intervention may result in unwanted side-effects. Indeed, sleep inducing drugs may also cause undesired carry-over effects, such as excessive sleepiness after sleep when wakefulness is required. Therefore, for sleep and alertness management in a military setting a combination of a short acting hypnotic drug and a fast acting stimulant drug are preferred.

In the present study some potentially useful sleep inducing drugs and alertness enhancing drugs have been tested in marmoset monkeys in order to assess their pharmacokinetics in this species. Also, the observed pharmacokinetics in marmoset monkeys were compared to those observed in humans in order to validate the usefulness of the marmoset monkey model for consecutive studies.

Three sleep-inducing (temazepam, zolpidem and zaleplon) and three wake-promoting (flumazenil, caffeine and modafinil) drugs were tested to determine their pharmacokinetics in marmoset monkeys. For sleep and alertness maintenance a combination of a short acting hypnotic drug (low  $t_{1/2}$ ) and a fast acting stimulant drug (low  $t_{C_{max}}$ ) may be necessary in short scenario's like rapid change between day- and night shift. Therefore, the pharmacokinetics of these drugs were investigated to determine whether they fulfill the prerequisites of short and fast action to allow the use of these drugs in aiding sleep and enhancing alertness during military service.

In order to attain this goal, sensitive and selective methods of sample preparation and analysis (HPLC and GC-MS) were developed. This was done by using high quality separation and detection methods, with the ideal internal standards and a good sample clean-up. Since there are different peak patterns in humans and the possible maximal volume of blood obtainable from marmoset monkeys is lower than in humans, the developed methods had to be optimized for the use of blood of marmoset monkeys.

Blood samples were obtained from non-anaesthetized marmoset monkeys. No anesthetic was used since there was no need for anesthesia as a result of a collection protocol which allowed for low-stress collections. More so, the anesthetic drug might interfere with the effects of the drugs under investigation. Often, canules are used for such sample collections, however in that case the animals have to be immobilized for the duration of the study. This immobilization could lead to excessive stress which might also interfere with the effects of the drugs. Therefore, in the present study small samples were taken from non-anaesthetized marmoset monkeys.

However, due to the limited number of sample collection times the obtained fits can only be considered as gross estimations of the pharmacokinetics of the drugs under investigation. Indeed, the 95% confidence limits of the fit parameters cover a large range around the fitted values (data not shown). Another analysis of the pharmacokinetics of temazepam, zolpidem and zaleplon in marmoset monkeys, using the same raw data [Kersten, 2002], showed some differences with the present study (see Table 18). However, it must be noted that this other analysis used a different method, and also only provided an estimation of the pharmacokinetics of these compounds.

Table 18 Comparison of the analyses results of the samples obtained from marmoset monkeys in the present study with those obtained by Kersten [2002].

	Temazepam		Zolpidem		Zaleplon	
	present study	Kersten [2002]	present study	Kersten [2002]	present study	Kersten [2002]
Dose (mg/kg)	15	15	3	3	10	10
$t_{1/2}$ (min)	46	76	117	76	33	48
$C_{max}$ (µg/ml)	0.22	0.24	0.35	0.47	0.32	0.33
$t_{Cmax}$ (min)	74	91	62	52	49	50

The main differences between these two types of analyses can be found in the elimination half-lives ( $t_{1/2}$ ) of these 3 drugs, which indicates that the results are estimations.

## 5.1 Comparing pharmacokinetics of marmosets with humans

Table 19 gives a quick overview of fit parameters of the curves describing the pharmacokinetics in marmoset monkeys of the drugs under investigation (flumazenil was not included since there was no curve fit). Also, in this table the human counterparts of these fit parameters, taken from the literature, are included.

Table 19 Comparison of the analyses results of the samples obtained from marmoset monkeys with those obtained in humans (results taken from literature).

Subject	Drug	Dose	$t_{1/2}$ (min)	$C_{max}$ (µg/ml)	$t_{Cmax}$ (min)
Marmoset	Temazepam	15 (mg/kg)	46	0.22	74
	Zolpidem	3 (mg/kg)	117	0.35	62
	Zaleplon	10 (mg/kg)	33	0.32	49
	Caffeine	30 (mg/kg)	595	16	79
	Modafinil	100 (mg/kg)	277	10.7	279
Human	Temazepam*	10 mg	720	0.23	72
	Zolpidem**	10 mg	120	0.125	102
	Zaleplon***	10 mg	62	0.037	46
	Caffeine****	5 mg	162-594	10	30
	Modafinil*****	200 mg	732	4.1	120

\* Jochemsen et al. (1983), \*\* Greenblatt et al. (1998), \*\*\* Sanchez Garcia *et al.* (2000), \*\*\*\* Blanchard and Sawers (1983), \*\*\*\*\* Wong *et al.* (1998).

In general it can be said that the smaller the primate the faster the metabolism [Gaulin and Konner, 1977]. But also (related) differences in pharmacokinetic mechanisms might be of importance, for example the species difference in metabolism of zaleplon [Kawashima *et al.*, 1999].

### 5.1.1 Sleep inducing drug: Temazepam

The time of the peak concentration ( $t_{Cmax}$ ) after temazepam in marmosets as obtained in the present study (74 min) equals the value that is observed in humans (72 min) [Jochemsen *et al.*, 1983]. However, in marmoset monkeys (present study as well as Kersten [2002]) the elimination half-life ( $t_{1/2}=46$ ) of temazepam is much lower than that observed in humans [Jochemsen *et al.*, 1983 ( $t_{1/2}=720$  min); Lockniskar and Greenblatt, 1990 ( $t_{1/2}=594$  min)], i.e. temazepam clearance is much quicker in marmoset monkeys than in humans. Interestingly, Schwarz [1979] demonstrated considerable interspecies

variation with respect to excretion and metabolite patterns in blood and urine after temazepam.

#### 5.1.2 *Sleep inducing drug: Zolpidem*

The elimination half-life ( $t_{1/2}$ ) of zolpidem in marmoset monkeys equals that observed in humans [Greenblatt *et al.*, 1998]. The time of the peak concentration ( $t_{C_{max}}$ ) after zolpidem in humans shows a relatively large inter-study variation [Greenblatt *et al.*, 1998 (102 min); Olubodun *et al.*, 2003 (60 min)]. The time of the peak concentration ( $t_{C_{max}}$ ) after zolpidem in marmosets as obtained in the present study (62 min), as well as in the Kersten [2002] study (52 min), is comparable to the results of Olubodun *et al.* [2003] (60 min).

#### 5.1.3 *Sleep inducing drug: Zaleplon*

The time of the peak concentration ( $t_{C_{max}}$ ) after modafinil in marmosets as obtained in the present study (49 min) equals the value that is observed in humans (46 min) [Rosen *et al.*, 1999; Sanchez Garcia *et al.*, 2000]. However, in marmosets (present study and Kersten [2002]) the elimination half-life ( $t_{1/2}$ ) of zaleplon is lower than that observed in humans [Rosen *et al.*, 1999; Sanchez Garcia *et al.*, 2000], i.e. zaleplon clearance is somewhat quicker in marmoset monkeys than in humans.

#### 5.1.4 *Alertness enhancing drug: Flumazenil*

In the present study no concentration-time course could be fitted for flumazenil due to detection problems. Interestingly, in humans an oral dose of 200 mg/kg results in a peak concentration of 255 ng/ml after 41 min [Roncari *et al.*, 1986]. The currently used method of analysis has a detection limit of 50 ng/ml, which would indicate that in humans a dose of at least 40 mg/kg (in case of a linear dose-concentration relationship) should be administered in order to detect flumazenil in plasma. In general, the peak concentrations of the drugs are higher in the marmoset monkeys, even after lower doses (see Table 19). Therefore, it is not unreasonable to expect a detectable level of flumazenil in plasma after the 15 mg/kg of flumazenil given to marmoset monkeys in the present study. Nevertheless, flumazenil could not be detected consistently in this study. Perhaps, the method used in this study was not sensitive enough for the detection of flumazenil in plasma. Possibly, the use of a LC-MS system for the detection of flumazenil in plasma could be beneficial.

#### 5.1.5 *Alertness enhancing drug: Caffeine*

The study of Blanchard and Sawers [1983] showed that in humans there is a substantial inter-subject variability in caffeine plasma half-lives (see Table 19). Nevertheless, the mean half-life of caffeine in plasma (278 min) observed in a study by Gelal *et al.* [2003] closely resembles the mean half-life observed in the Blanchard and Sawers [1983] study (270 min). Interestingly, the observed plasma half-life of caffeine in marmosets (595 min) fits within the range observed in humans [Blanchard and Sawers, 1983]. The observed time of the peak concentration ( $t_{C_{max}}$ ) after caffeine in marmosets (79 min) is later than that observed in humans (30 min) [Blanchard and Sawers, 1983].

The present study also demonstrated a useful protocol for obtaining long-lasting stable caffeine concentrations in marmosets over time, by orally administering caffeine in a dose of 30 mg/kg and 5 mg/kg after 4 hours followed by 10 mg/kg after 8 hours. By using such a protocol toxic accumulation of peak levels of caffeine can be prevented as well as changes in the caffeine concentration in plasma. This will then facilitate a reliable steady state effect on a behavioral level, i.e. consistency in alertness levels will

be facilitated. However, some tolerance to caffeine effects has been reported [reviewed in Dews *et al.*, 2002]. Also, caffeine is a substance that is frequently used on a non-medical basis (i.e. coffee), which might complicate the regulation of dosing in humans.

#### 5.1.6 Alertness enhancing drug: Modafinil

The time of the peak concentration ( $t_{C_{max}}$ ) after modafinil in marmosets as obtained in the present study (279 min) is higher than the value that is observed in humans (120 min) [Wong *et al.*, 1998; Wong *et al.*, 1999; review in Robertson and Hellriegel, 2003]. Also, the elimination half-life ( $t_{1/2}$ ) of modafinil differs between marmoset monkeys (277 min; in this study) and humans (732 min) [Wong *et al.*, 1998; Wong *et al.*, 1999; review in Robertson and Hellriegel, 2003]. Accordingly, similar species differences in the pharmacokinetics of modafinil have been observed in another study [Moachon *et al.*, 1996].

## 5.2 Pharmacokinetics and sleep- and alertness management

Any pharmacological intervention may result in unwanted side-effects. Therefore, for sleep and alertness management in a military setting a combination of a short acting (rapid clearance; low  $t_{1/2}$ ) hypnotic drug and a fast acting (quick absorption; low  $t_{C_{max}}$ ) stimulant drug are preferred. In order to provide a quick overview of the absorption and clearance properties of the tested drugs, the drugs are placed in rank order according to their  $t_{C_{max}}$  and  $t_{1/2}$  values. Also, the observed pharmacokinetics in marmoset monkeys are compared to those observed in humans in order to validate the usefulness of the marmoset monkey model for consecutive studies.

#### 5.2.1 The time of the peak concentration ( $t_{C_{max}}$ ): absorption

The present study demonstrated the following orders in **marmoset monkeys** (see Table 19), when placing the time till reaching the peak concentration of the drugs from quick to slow:

- the sleep inducing drugs: zaleplon<zolpidem<temazepam;
- the alertness enhancing drugs: caffeine<modafinil.

In **humans** (see Table 19 and Section 5.1.1.2.) the following orders were observed:

- the sleep inducing drugs: zaleplon< zolpidem<sup>4</sup>/temazepam;
- the alertness enhancing drugs: caffeine<modafinil.

#### 5.2.2 The elimination half-life ( $t_{1/2}$ ): clearance

The present study demonstrated the following orders in **marmoset monkeys** (see Table 19), when placing the elimination half-life of the drugs from quick to slow:

- the sleep inducing drugs: zaleplon<temazepam<zolpidem;
- the alertness enhancing drugs: modafinil<caffeine.

In **humans** (see Table 19) the following orders were observed:

- the sleep inducing drugs: zaleplon<zolpidem<temazepam;
- the alertness enhancing drugs: caffeine<modafinil.

The observed differences in these orders of  $t_{1/2}$  values, between marmoset monkeys and humans are most likely due to the large difference between these species in the observed elimination half-lives after temazepam and modafinil.

<sup>4</sup> Due to the large inter-study variability in absorption of zolpidem in humans, the true overall  $t_{C_{max}}$  value most likely lies within the range of similarity to the absorption of temazepam in humans.

## 6 Conclusion

In the present study some potentially useful sleep inducing drugs and alertness enhancing drugs have been tested in marmoset monkeys in order to assess their pharmacokinetics in this species. Also, the observed pharmacokinetics in marmoset monkeys were compared to those observed in humans in order to validate the usefulness of the marmoset monkey model for consecutive studies.

In literature described research in human showed that among the selected hypnotics zaleplon leads to the most rapid absorption and also the fastest clearance, and among the selected alertness enhancing drugs, caffeine lead to a faster absorption as compared to modafinil. Despite some differences in general pharmacokinetics between the species, the present study also demonstrated the fastest absorption as well as the fastest clearance after zaleplon in marmoset monkeys. Also, the quick absorption of caffeine as compared to modafinil was observed in marmoset monkeys similar to the findings in human.

In conclusion, the present study shows that the marmoset monkey model is a valid model for measuring the pharmacokinetic effects of sleep inducing and alertness enhancing drugs, at least in the sense of a basis of pharmacological comparison. Furthermore, the present study indicates that zaleplon and caffeine might possess the most favourable pharmacokinetics for sleep- and alertness management.

However, despite possessing the most favourable pharmacokinetics for sleep- and alertness management, it does not necessarily mean that these two drugs also have the most favourable effects on performance. Therefore, in consecutive reports [TNO-DV 2006 A269, TNO-DV 2006 A270, TNO-DV 2006 A271] the behavioral effects of the selected drugs are discussed [Philippens *et al.* 2006].

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## 8 Signature

Rijswijk, October 2006

TNO Defence, Security and Safety

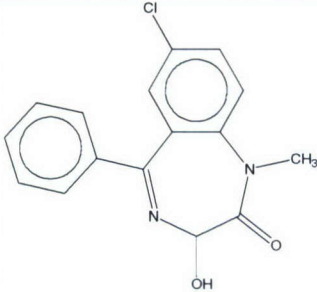
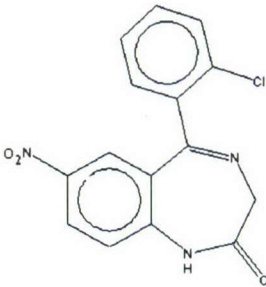
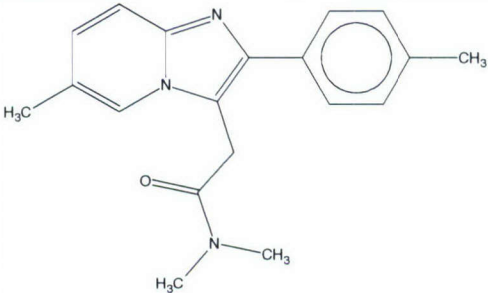
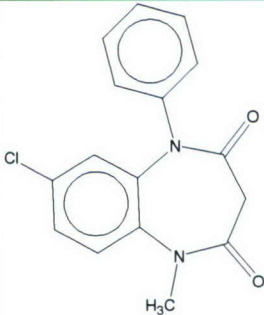
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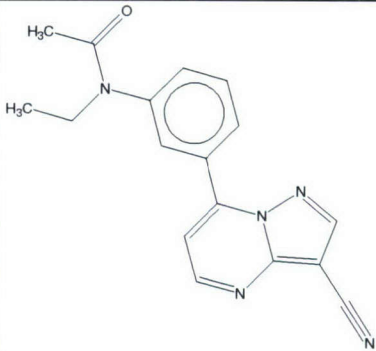

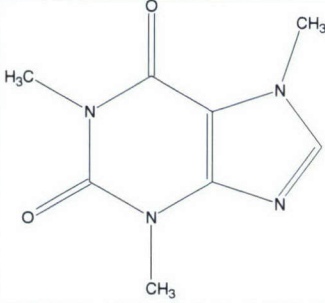
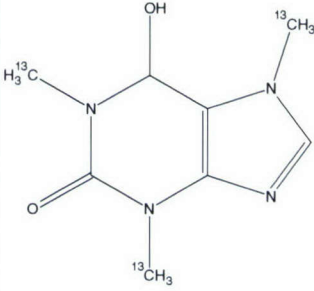
J. Schaafsma, MSc  
Group leader

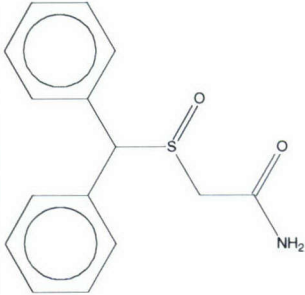
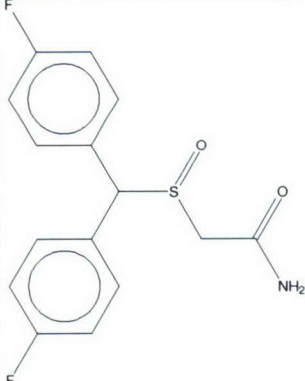
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Dr I.H.C.H.M. Philippens  
Project leader/Author

## A Structural information of target compounds and internal standards

Name	IUPAC chemical name	Structure, chemical formula, Molecular weight and CAS number
<b>Temazepam</b>	3-hydroxy diazepam, 7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one	 <p><chem>Cc1c(O)c2c(c1)c3cc(Cl)ccc3n2C4=CC=CC=C4</chem>  <math>C_{16}H_{13}ClN_2O_2</math>, MW 300.7 g/mol, CAS 846-50-4</p>
<b>Clonazepam</b>	5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one	 <p><chem>O=C1CNc2cc3c(cc21)cc(cc3[N+](=O)[O-])C4=CC=C(C=C4)C5=CC=CC=C5Cl</chem>  <math>C_{15}H_{10}ClN_3O_3</math>, MW 315.7, CAS1622-61-3</p>
<b>Zolpidem</b>	<i>N,N</i> ,6-trimethyl-2- <i>p</i> -tolylimidazo[1,2- <i>a</i> ]pyridine-3-acetamide	 <p><chem>Cc1ccc(cc1)C2=C3C(=CN2)C=C(C=C3)C(C4=CC=CC=C4C)C(=O)N(C)C</chem>  <math>C_{19}H_{21}N_3O</math>, MW 307.4 g/mol, CAS 82626-48-0</p>
<b>Clobazam</b>	7-Chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4(3H,5H)-dione	 <p><chem>Cc1c2c(c3cc(Cl)ccc3n2C4=CC=CC=C4)C(=O)CC5=CC=CC=C5C1=O</chem>  <math>C_{16}H_{13}ClN_2O_2</math>, MW 300.7, CAS 22316-47-8</p>

Name	IUPAC chemical name	Structure, chemical formula, Molecular weight and CAS number
<b>Zaleplon</b>	N-[3-(cyanopyrazolo[1,5-a]pyrimidin-7-yl)-N-ethylacetamide	 <p>C<sub>17</sub>H<sub>15</sub>N<sub>5</sub>O, MW 305.3, CAS 151319-34-5</p>
<b>Flumazenil</b>	ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate	 <p>C<sub>15</sub>H<sub>14</sub>FN<sub>3</sub>O<sub>3</sub>, MW 303.3 g/mol, CAS 787558-81-4</p>
<b>Caffeine</b>	1,3,7-trimethyl-2,6-dioxo-1,2,3,6-tetrahydropurine	 <p>C<sub>8</sub>H<sub>10</sub>N<sub>4</sub>O<sub>2</sub>, MW 194.2 g/mol CAS 58-08-2</p>
<b>caffeine-trimethyl-<sup>13</sup>C<sub>3</sub>, <sup>13</sup>C<sub>3</sub>-CA</b>		 <p>C<sub>8</sub>H<sub>10</sub>N<sub>4</sub>O<sub>2</sub>, MW 197.2 g/mol CAS 78072-66-9</p>

Name	IUPAC chemical name	Structure, chemical formula, Molecular weight and CAS number
<b>Modafinil</b>	2-[(diphenylmethyl)sulfinyl] acetamide	 <chem>CC(=O)NCCS(=O)C(c1ccccc1)c2ccccc2</chem> C <sub>15</sub> H <sub>15</sub> NO <sub>2</sub> S, MW 273.35, CAS 68693-11-8
<b>Modafinil with 2 fluoro groups</b>	[bis-(4-fluor-phenyl)sulfinyl]acetic acid	 <chem>CC(=O)NCCS(=O)C(c1ccc(F)cc1)c2ccc(F)cc2</chem> C <sub>15</sub> H <sub>13</sub> F <sub>2</sub> NO <sub>2</sub> S, MW 309.3, CAS N/A

## B Materials and chemicals

### *Temazepam*

- Temazepam (7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, C<sub>16</sub>H<sub>13</sub>ClN<sub>2</sub>O<sub>2</sub>, M<sub>w</sub> 300,74 g/mol, Duchefa).
- Clonazepam (5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one, Duchefa).
- Milli-Q water.
- Methanol, Fluka HPLC-grade, >99.9%.
- Acetonitril, Aldrich 99.93%+ HPLC grade.
- o-Phosphoric acid (Merck, 85%, p.a.).
- Triethylamine (TEA), (Aldrich, p.a.).
- Sodium Hydroxyde, p.a, Merck.
- Nexux SPE, LRC/10 cc/60 mg, (crosslinked copolymer, based on polystyrene/methacrylate), part. nr. 1211-3101, Varian.
- 50 mM phosphate buffer with 0.5% (v) TEA: 5.0 ml TEA, 4.5 ml o-phosphoric acid, 4.0 ml 6M NaOH, 800 ml MQ, adjust to pH 3.5 and adjust the volume to 1000 ml.

### *Zolpidem*

- Zolpidem (*N,N*,6-trimethyl-2-*p*-tolylimidazo[1,2-*a*]pyridine-3-acetamide), Sigma, Z103.
- Clobazam (7-Chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4(3H,5H)-dione), Sigma.
- Milli-Q water.
- Methanol, Fluka HPLC-grade, ≥99.8%.
- Acetonitril, Aldrich 99.93%+ HPLC grade.
- Sodium Hydroxyde, p.a, Merck.
- Disodiumhydrogenphosphate.2H<sub>2</sub>O (>99%), Merck.
- Potassium dihydrogenphosphate (>99%), Merck.
- Dichloormethaan (>99%), Merck.
- n-Pentaaan (± 99%), Merck.
- 25 mM phosphate buffer: 585 ml of 4.45 g Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O/l and 415 ml of 3.04 g KH<sub>2</sub>PO<sub>4</sub>/l, adjust to pH 7.0.

### *Zaleplon*

- Zaleplon (N-[3-(3-cyanopyrazolo[1,5-*a*]pyrimidin-7-yl)phenyl]-N-ethylacetamide), TNO-PML, pure compound isolated from Sonata® capsules, purity 94 mol% (NMR).
- Zolpidem (*N,N*,6-trimethyl-2-*p*-tolylimidazo[1,2-*a*]pyridine-3-acetamide), Sigma, Z103.
- Milli-Q water.
- Sodium Hydroxyde, p.a, Merck.
- Methanol, Fluka HPLC-grade, ≥99.8%.
- Acetonitril, Aldrich 99.93%+ HPLC grade.
- Disodiumhydrogenphosphate.2H<sub>2</sub>O (>99%), Merck.
- Potassium dihydrogenphosphate (>99%), Merck.
- Human plasma, heperanized.
- 25 mM phosphate buffer: 585 ml of 4.45 g Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O/l and 415 ml of 3.04 g KH<sub>2</sub>PO<sub>4</sub>/l, adjust to pH 7.0.

*Flumazenil*

- Flumazenil, La Roche, lot 406004.
- Clonazepam (5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one, Duchefa).
- Milli-Q water.
- Methanol, Fluka HPLC-grade, >99.9%.
- o-Fosforzuur (Merck, 85%, p.a.).
- Diethylether, Labscan, >98%.
- Dichloromethane, Merck, zA.
- Triethylamine (TEA), (Aldrich, p.a.).
- Human plasma, heperanized.
- Disodium hydrogenphosphate.12H<sub>2</sub>O (>99%), Merck.
- Sodiumdihydrogen phosphate. (>99%), Merck.
- Sodium Hydroxyde, p.a, Merck.
- 25 mM phosphate buffer: Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O 4.48 g and 1.72 g NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O, adjust to pH 7.0.
- phosphate buffer pH 9: 100 ml phosphate buffer pH 7, adjust pH to 9 with 1M NaOH.

*Caffeine*

- Caffeine, p.a., Sigma.
- Caffeine-trimethyl-<sup>13</sup>C<sub>3</sub> (Sigma).
- Milli-Q water.
- Methanol, Fluka HPLC-grade, >99.9%.
- Sodium Hydroxyde, p.a, Merck.
- Nexux SPE, LRC/10 cc/60 mg, Varian.
- BSA: albumine bovine fraction V pH 7 purity 98% ICN.
- Acetic acid anhydrous, Merck, pa.
- Nexux SPE, LRC/10 cc/60 mg, (crosslinked copolymer, based on polystyrene/methacrylate), part. nr. 1211-3101, Varian.
- Acetic acid buffer 0.1 M: 0.6 ml acetic acid and 100 ml MQ, adjust to pH 5.0 with 12 M NaOH.
- 5% blank matrix of BSA: 5 g BSA + 95 ml MQ.

*Modafinil*

- Modafinil, 2-[(diphenylmethyl)sulfinyl] acetamide\*.
- difluor-modafinil (Internal standard), [bis-(4-fluor-phenyl)sulfinyl]acetic acid\*\*.
- Nexus SPE 6ml 200mg, (crosslinked copolymer, based on polystyrene/methacrylate), Varian part. nr. 1210-3102.
- Milli-Q water.
- Ethylacetate, analytical grade, Merck.
- Sodium hydroxide, pa, Merck.
- Sodium acetate-trihydrate, pa, Merck.
- Acetic acid (glacial), pa, Merck.
- Orthophosphoric acid, pa, Merck.
- IPA (propanol-2), pa, Merck.
- Acetone, technical grade, Merck.
- Dry ice.
- Acetonitrile HPLC grade, Biosolve.
- Nitrogen.

\* and \*\*, a kind gift from P. Burnat, Laboratoire de Biochimie et Toxicologie Cliniques, Paris, France.

## C Some representative chromatograms of the HPLC and GC-MS analysis of the investigated drugs

Examples of chromatograms obtained under HPLC and GC-MS conditions as mentioned in 3.5 and 3.6 are shown in the figures below:

- C.1 Temazepam;
- C.2 Zolpidem;
- C.3 Zaleplon;
- C.4 Flumazenil;
- C.5 Caffeine;
- C.6 Modafinil.

Conditions and results are summarized.

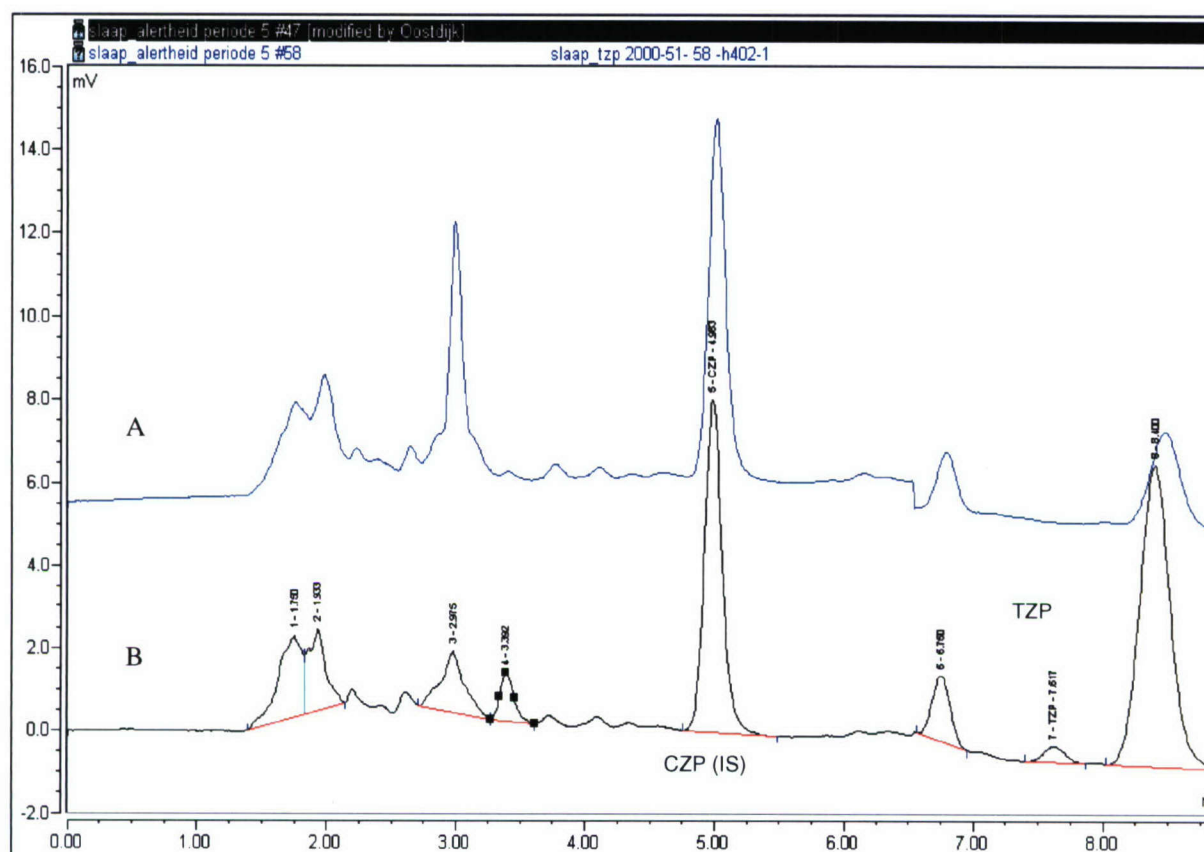


Figure C.1 Temazepam.

HPLC analysis of blood sample extracts obtained from a marmoset (animal 4) after an oral administration of a dose of 15.4 mg/kg temazepam:

- a) blank blood sample (0 min);
- b) sample taken 243 min after oral administration (150 µl).

The concentration of temazepam in the blood sample was 70 ng/ml, using the internal standard (clonazepam) method and calculated on a calibration curve based on peak height ratio.

X-axis: Time (min), Y-axis: UV signal (mV).

#### HPLC conditions (configuration 1):

##### Sample:

injection volume: 50 µl.

##### Eluents:

MeOH/ACN/phosphate buffer pH 3.5 with 0.5 vol% TEA (50/10/40 vol%).

##### Column:

guard Inertsil ODS 10 µm 7.5 x 4.6 mm and analytical column LiChrospher RP-18 d<sub>p</sub> 3 µm 150 x 4.6 mm., temperature column 35 °C, flow 1 ml/min.

##### Detection:

UV detection: 0-6.5 min 309 nm en 6.5-9 min on 230 nm.

##### Retention times:

Temazepam (TZP): 7.62 min (target compound).

Clonazepam (CZP): 4.98 min (internal standard).

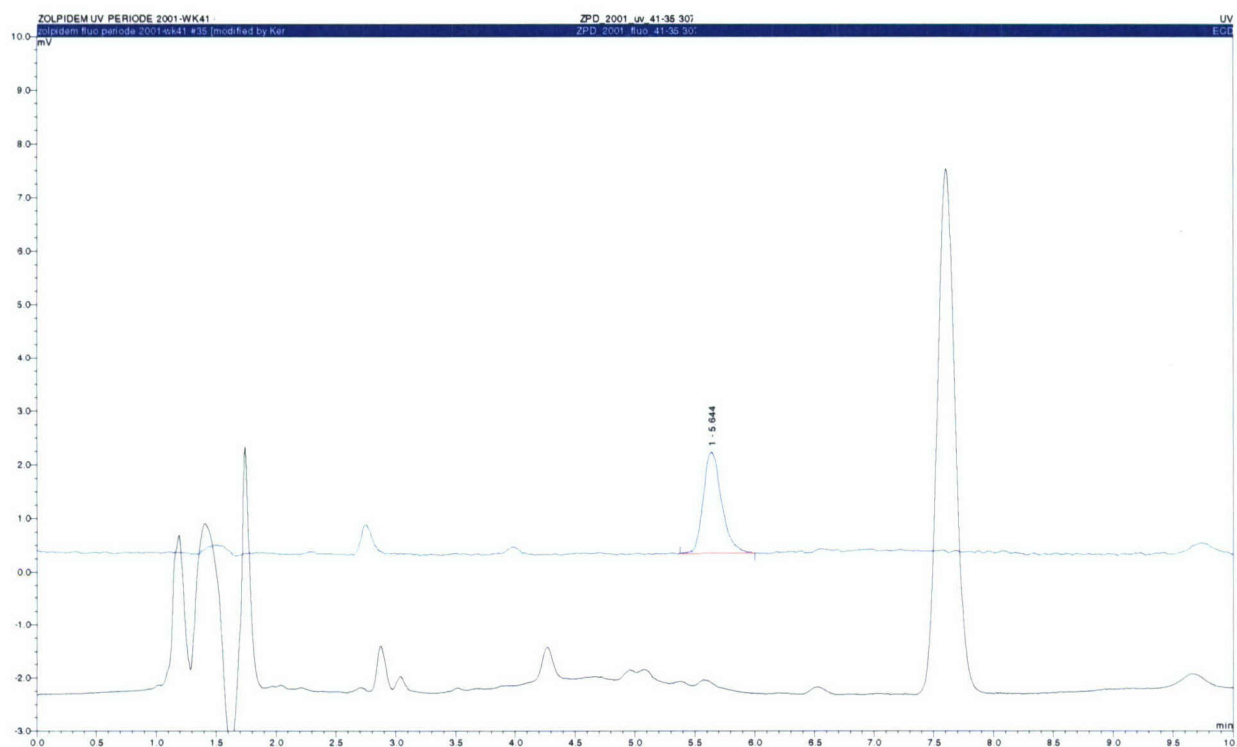


Figure C.2 Zolpidem.

HPLC analysis of plasma extracts obtained from an anaesthetized marmoset (animal 1) after an oral administration of a dose of 2.9 mg/kg zolpidem. The blood sample was taken at 246 min after oral administration (175 µl blood, 50 µl plasma used for analysis).

- a) Fluorescence signal (zolpidem);
- b) UV signal (clobazam).

The concentration of zolpidem in the plasma sample was 66 ng/ml, using the internal standard (clobazam) method and calculated on a calibration curve based on peak area ratio.

X-axis: Time (min), Y-axis: Fluorescence and UV signal (mV).

#### HPLC conditions (configuration 1):

##### Sample:

injection volume: 40 µl.

##### Eluents:

phosphate buffer (pH7)/ACN (55:45) v/v.

##### Column:

guard Inertsil ODS 10 µm 7.5 x 4.6 mm and analytical column C18 ALLTIMA column: 150 mm x 4.6 mm, d<sub>p</sub> 5 µm, temperature column 30 °C, flow 1 ml/min.

##### Detection:

Fluorescence detection at excitation 254 nm and emission 390 nm (gain 10) and UV detection: 230 nm.

##### Retention times:

Zolpidem (ZPD): 5.64 min (target compound).

Clobazam (CBZ): 7.49 min (internal standard).

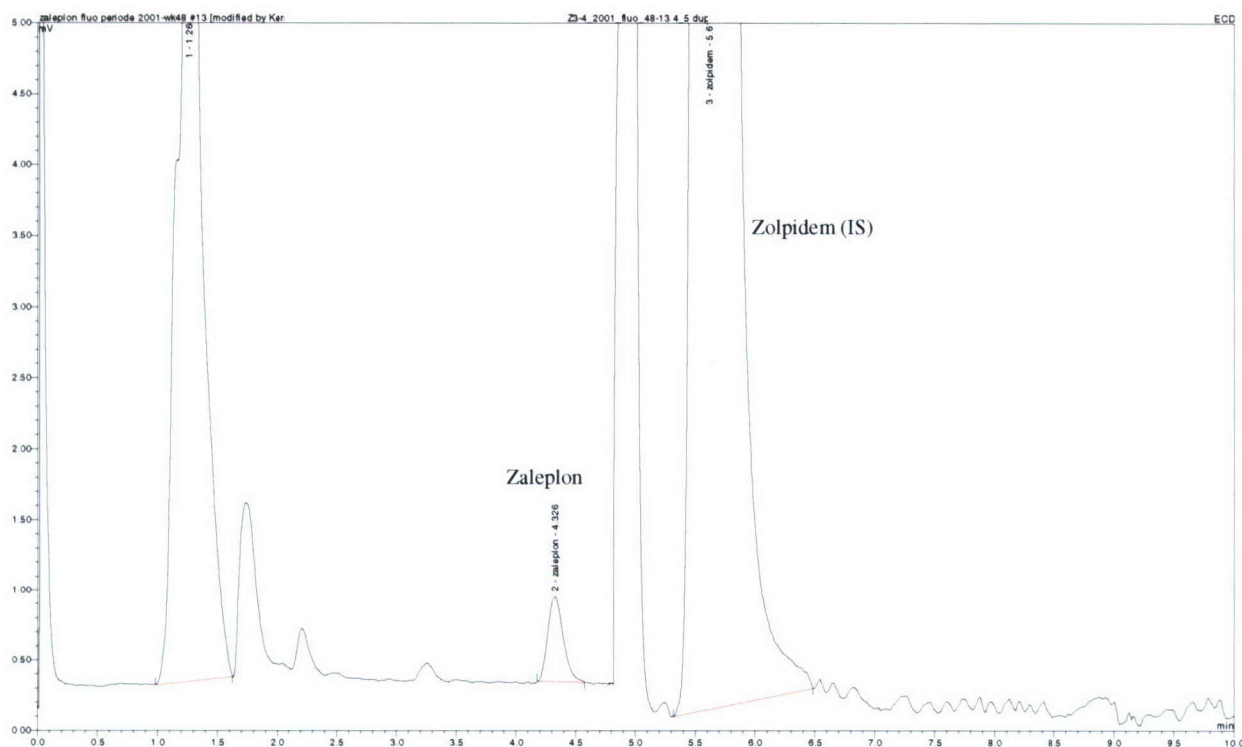


Figure C.3 Zaleplon.

HPLC analysis of plasma extracts obtained from a marmoset (animal 4) after an oral administration of a dose of 10 mg/kg zolpidem. The blood sample was taken at 244 min after oral administration (200 µl blood, 50 µl plasma used for analysis).

The concentration of zaleplon in the plasma sample was 12 ng/ml, using the internal standard (zolpidem) method and calculated on a calibration curve based on peak area ratio.

X-axis: Time (min), Y-axis: Fluorescence signal (mV).

#### HPLC conditions (configuration 1):

##### Sample:

injection volume: 20 µl.

##### Eluents:

phosphate buffer (pH7)/ACN (55:45) v/v.

##### Column:

guard Inertsil ODS 10 µm 7.5 x 4.6 mm and analytical column C18 ALLTIMA column: 150 mm x 4.6 mm, d<sub>p</sub> 5 µm, temperature column 30 °C, flow 1 ml/min.

##### Detection:

Fluorescence detection (0-5 min) at excitation 345 nm/emission 460 nm and 5-10 min at excitation 254 nm/emission 390 nm (gain 10).

##### Retention times:

Zaleplon (ZAL): 4.33 min (target compound).

Zolpidem (ZPD): 5.61 min (internal standard).

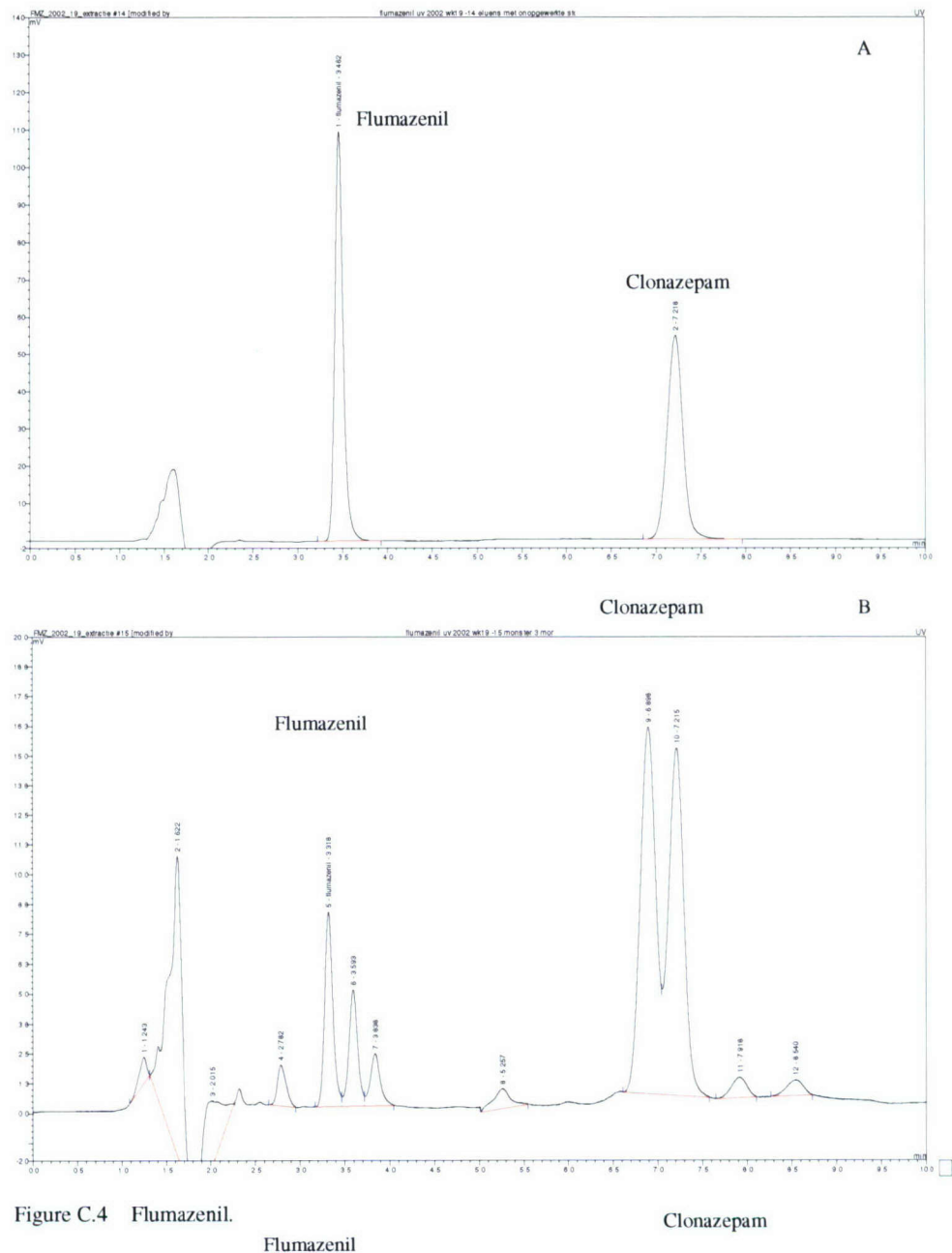


Figure C.4 Flumazenil.

HPLC analysis of plasma extracts obtained from a marmoset (animal 1) after an oral administration of a dose of 15 mg/kg flumazenil. The blood sample was taken at 56 min after oral administration (200  $\mu$ l blood, 115  $\mu$ l plasma used for analysis).

- a standard solution of flumazenil and clonazepam (no peaksplitting, no sample clean-up): 6.6  $\mu$ g FLU/ml eluens and 5.9  $\mu$ g CZP/ml eluens.
- 56 min sample of animal 1.
- a standard solution of flumazenil and clonazepam (with peaksplitting after sample clean-up): 660 ng FLU/ml eluens and 5.9  $\mu$ g CZP/ml eluens.

The concentration of flumazenil in the plasma sample was 730 ng/ml, using the internal standard (Clonazepam) method and calculated on a calibration standard based on peak area ratio (also with peaksplitting).

X-axis: Time (min), Y-axis: UV signal (mV).

**HPLC conditions (configuration 1):**Sample:

injection volume: 40 µl.

Eluents:

phosphate buffer + TEA (0.5%) v/v (pH7)/ACN (60:40) v/v.

Column:

guard Inertsil ODS 10 µm 7.5 x 4.6 mm and analytical column LiChrospher RP-18 d<sub>p</sub> 3 µm 150 x 4.6 mm.,  
temperature column 35 °C, flow 1 ml/min.

Detection:

UV detection: 245 nm.

Retention times:

Flumazenil (FLU): appr. 3.3 min (target compound) \*.

Clonazepam (CZP): appr. 6.9 min (internal standard) \*.

\* Because of peaksplitting after sample clean up (animal 1), the retention time differs, as shown in a) with normal peak shape. This gives a retention time of 3.46 and 7.22 min for FLU and CZP, respectively. Sample clean-up with new solutions solved this problem, but no Flumazenil was detected.

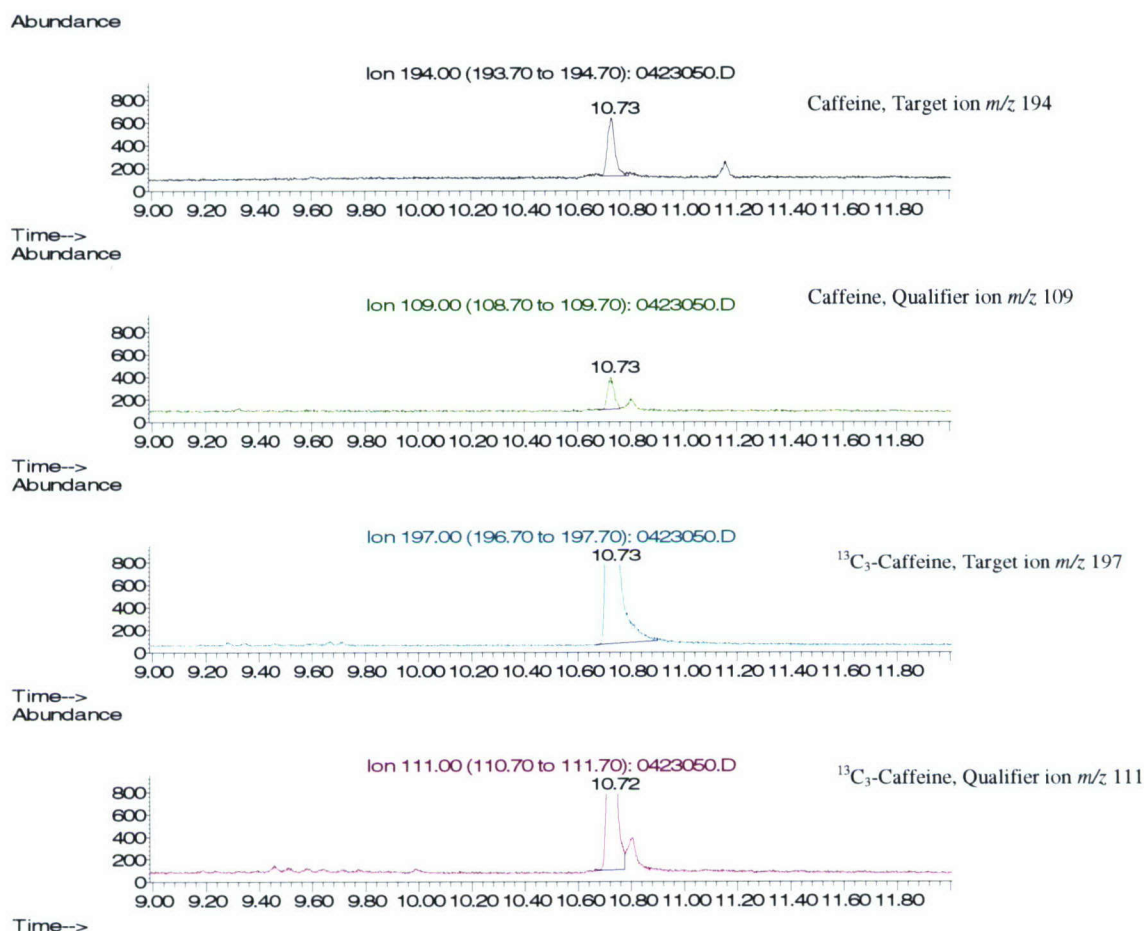


Figure C.5 Caffeine.

GC-MS analysis of plasma extracts obtained from a marmoset (animal 4) after an oral administration of a dose of 30 mg/kg caffeine. The blood sample was taken at 145 min after oral administration (approximately 100 µl blood, 5 µl plasma used for analysis).

The concentration of caffeine in the plasma sample was 9.6 µg/ml, using the internal standard ( $^{13}\text{C}_3$ -caffeine) method and calculated on a calibration curve based on peak height ratio.

X-axis: Time (min), Y-axis: Abundance (AU).

#### GC-MS conditions :

##### Sample:

injection volume: 1 µl 270 °C splitless time 0.25 min @ 150 ml/min.

##### Carrier gas:

Helium, constant flow 2.0 ml/min.

##### Column:

RTX-5 Restek, 30 m x 0.25 mm i.d., df 0.25 µm.

oven: 100 °C(1')---20°C/min--->260 °C(3').

##### Detection:

MSD, SIM mode, m/z 109 – 111 – 194 – 197 (dwell time 15 ms).

##### Retention times:

Caffeine (CA): 10.73 min (target compound).

$^{13}\text{C}_3$ -caffeine ( $^{13}\text{C}_3$ -CA): 10.73 min (internal standard).

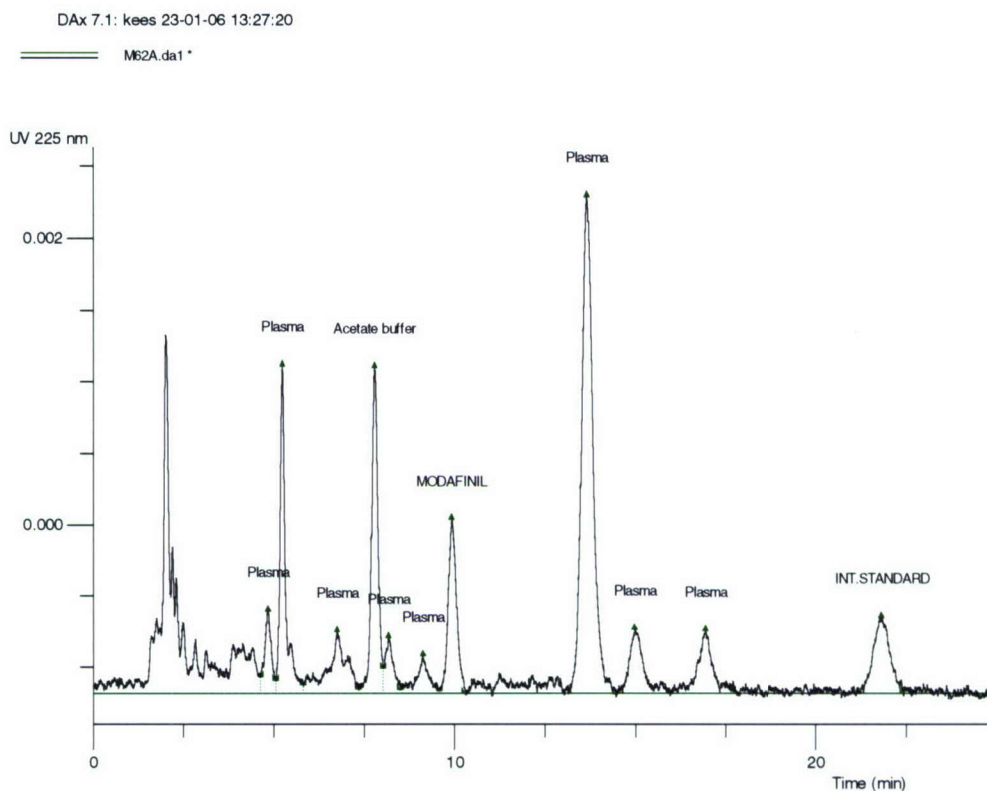


Figure C.6 Modafinil.

HPLC analysis of plasma extracts obtained from a marmoset (animal 6) after an oral administration of a dose of 100 mg/kg modafinil. The blood sample was taken at 70 min after oral administration (100-200  $\mu$ l blood, 50  $\mu$ l plasma used for analysis).

The concentration of modafinil in the plasma sample was 3.82  $\mu$ g/ml, using the internal standard method and calculated on a calibration curve based on peak area ratio.  
X-axis: Time (min), Y-axis: UV signal (mV).

#### HPLC conditions (configuration 2):

##### Sample:

injection volume: 20  $\mu$ l.

##### Eluents:

ACN/0.05M phosphate buffer pH 2.6 (28.5/71.5) v/v.

##### Column:

guard ODS 10  $\mu$ m 20 x 4.6 mm and analytical column SunFire C8  $d_p$  5  $\mu$ m 150 x 4.6 mm, temperature column 30 °C, flow 1 ml/min.

##### Detection:

UV detection: 225 nm.

##### Retention times:

Modafinil (MOD): 10.0 min (target compound) \*.

[bis-(4-fluor-phenyl)sulfinyl]acetic acid: 22.0 min (internal standard).

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15. ABSTRACT (MAXIMUM 200 WORDS (1044 BYTE))
The use of sleep-inducing and wake-promoting drugs can have direct usefulness in crew endurance plans. However, any pharmacological intervention may result in unwanted side-effects. Therefore, for sleep and alertness management in a military setting a combination of a short acting hypnotic drug and a fast acting stimulant drug are preferred. Therefore, the pharmacokinetics of the hypnotic drugs temazepam, zolpidem and zaleplon, and the alertness enhancers flumazenil, caffeine and modafinil were investigated to determine whether they fulfill the prerequisites of short and fast action to allow the use of these drugs during military service. In order to attain this goal, sensitive and selective methods of sample preparation and analysis (HPLC and GC-MS) were developed by using high quality separation and detection methods. The present study shows that the marmoset monkey is a valid model for measuring the pharmacokinetic effects of fast sleep inducing and alertness enhancing drugs. Furthermore, the results indicate that, in marmosets as well as in human, zaleplon and caffeine might possess the most favourable pharmacokinetics for sleep- and alertness management. However, despite possessing the most favourable pharmacokinetics for sleep- and alertness management, it does not necessarily mean that these two drugs are also the most effective drugs.

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